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**(54) Title:** STABLE POLYMERIZED HEMOGLOBIN BLOOD-SUBSTITUTE

**(57) Abstract**

A method for producing a stable polymerized hemoglobin blood-substitute from blood characterized in the use of a chromatographic column, is disclosed. The method of this invention includes mixing blood with an anticoagulant to form a blood solution, washing the red blood cells in the blood solution and then separating the washed red blood cells from the white blood cells. This method also includes disrupting the red blood cells to release hemoglobin and form a hemoglobin solution, which is then treated by high performance liquid chromatography to form a hemoglobin eluate. The hemoglobin eluate is then deoxygenated, contacted with a first reducing agent t form an oxidation-stabilized deoxygenated hemoglobin solution, and mixed with a cross-linking agent to form a polymerization reaction mixture, which is then polymerized. The polymerized hemoglobin solution is then diafiltered with a physiologic solution and with a reducing agent, whereby the polymerized hemoglobin solution is made physiologically acceptable, and whereby the reducing agent scavenges xygen, to form a stable polymerized hemoglobin blood-substitute, which is then packaged and stored in an atmopshere substantially free of oxygen. Compositions made by the methods are also disclosed, as are methods of therapeutically, or prophylactically, treating a vertebrate to increase tissue oxygenation, or prevent oxygen depletion, in tissue of the vertebrate.

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# STABLE POLYMERIZED HEMOGLOBIN BLOOD-SUBSTITUTE

## Background of the Invention

There exists a need for a blood-substitute to treat or prevent hypoxia resulting from blood loss (e.g, from acute hemorrhage or during surgical operations), resulting from anemia (e.g., pernicious anemia or sickle cell anemia), or resulting from shock (e.g, volume deficiency shock, anaphylactic shock, septic shock or allergic shock).

The use of blood and blood fractions as in these capacities as a blood-substitute is fraught with disadvantages. For example, the use of whole blood often is accompanied by the risk of transmission of hepatitis-producing viruses and AIDS-producing viruses which can complicate patient recovery or result in patient fatalities. Additionally, the use of whole blood requires blood-typing and cross-matching to avoid immunohematological problems and interdonor incompatibility.

Human hemoglobin, as a blood-substitute, possesses osmotic activity and the ability to transport and transfer oxygen, but it has the disadvantage of rapid elimination from circulation by the renal route and through vascular walls, resulting in a very short, and therefore, a typically unsatisfactory half-life. Further, human hemoglobin is also frequently contaminated with toxic levels of endotoxins, bacteria and/or viruses.

Non-human hemoglobin suffers from the same deficiencies as human hemoglobin. In addition, hemoglobin from non-human sources is also typically contaminated with proteins, such as antibodies, which could cause an immune system response in the recipient.

Previously, at least four other types of blood-substitutes have been utilized, including

perflu rochemicals, synthesized hemoglobin analogues, liposome-encapsulated hemoglobin, and chemically-modified hemogl bin. However, many of these blood-substitutes have typically had short intravascular retention times, being removed by the circulatory system as foreign substances or lodging in the liver, spleen, and other tissues. Also, many of these blood-substitutes have been biologically incompatible with living systems.

Thus, in spite of the recent advances in the preparation of hemoglobin-based blood-substitutes, the need has continued to exist for a blood-substitute which has levels of contaminants, such as endotoxins, bacteria, viruses, phospholipids and non-hemoglobin proteins, which are sufficiently low to generally prevent an immune system response and any toxicological effects resulting from an infusion of the blood-substitute. In addition, the blood-substitute must also be capable of transporting and transferring adequate amounts of oxygen to tissue under ambient conditions and must have a good intravascular retention time.

Further, it is preferred that the blood-substitute 1) has an oncotic activity generally equivalent to that of whole blood, 2) can be transfused to most recipients without cross-matching or sensitivity testing, and 3) can be stored with minimum amounts of refrigeration for long periods.

## Summary of the Invention

The present invention relates to a method for producing a stable polymerized hemoglobin blood-substitute from whole blood, employing a chromatographic column. The invention also relates to a method of preserving the stability of a hemoglobin blood substitute in an atmosphere substantially free of oxygen. The invention further relates to a composition of matter

comprising a stable polymerized hemoglobin product, as well as stable solution comprising polymerized hemoglobin and a reducing agent.

In one embodiment, the method involves mixing blood with an anticoagulant to form a blood solution and then washing the red blood cells in the blood solution to separate small plasma proteins from the red blood cells. The method also includes the steps of separating the washed red blood cells from the white blood cells and then disrupting the red blood cells to release hemoglobin and form a hemoglobin solution. Non-hemoglobin components in the hemoglobin solution are subsequently separated from the hemoglobin solution by molecular weight fractionation on 100 kD and 30 kD nominal molecular weight cut-off ultrafilters and high performance liquid chromatography to form a hemoglobin eluate. The hemoglobin eluate is then deoxygenated and subsequently contacted with a reducing agent to form an oxidation-stabilized deoxygenated hemoglobin solution, which is subsequently mixed with a cross-linking agent to form a polymerization reaction mixture. The polymerization reaction mixture is then stabilized and diafiltered with a physiologic solution and with a reducing agent, whereby the polymerized hemoglobin solution is made physiologically acceptable, and whereby the reducing agent scavenges trace levels of oxygen, thereby forming said stable polymerized hemoglobin blood-substitute.

The blood-substitutes of the invention can be used in a method for increasing tissue oxygenation in tissue of a vertebrate, while the tissue has reduced red blood cell flow, and wherein the vertebrate has a normovolemic blood volume and at least a normal systemic vascular resistance, by introducing into the circulatory system of vertebrate, at least one dose of hemoglobin.

The advantages of this invention ar numerous. One advantage is that the hemoglobin produced by the method of this invention has a greater degree of purity than previous methods. It is substantially free of even recalcitrant protein materials such as carbonic anhydrase. Thus, the hemoglobin derived from one species can be successfully used in a different species as a blood-substitute without the recipient species suffering significant side effects.

The stable polymerized hemoglobin blood-substitute, produced from the method of this invention, can be manufactured from bovine blood which is available in large volumes and low cost. Bovine hemoglobin has the further advantage of having a physiologically relevant oxygen binding curve without the need for chemical modification reagents such as pyridoxal-5-phosphate or other chemicals that are required to modify the oxygen affinity of human hemoglobin.

Further, the polymerized hemoglobin is also produced in higher yields, with lower levels of non-stabilized hemoglobin, than in previous methods.

In addition, the blood-substitute produced and stored by the methods of the invention has a greater degree of purity and longer shelf life. The blood substitute is stable at room temperature for periods up to two years or more. The blood-substitute has a relatively low oxygen affinity, an increased intravascular retention time, and a suitable oncotic pressure.

This invention has a further advantage in reducing the probability and extent of tissue and/or organ hypoxia, and of possible tissue necrosis, resulting from at least a partial reduction in RBC flow. Another advantage is improved survivability for a vertebrate suffering from a significant reduction in RBC flow to a

vital organ or portion thereof. This invention also allows the performance of invasive procedures, which require restriction of RBC flow, without significantly reducing oxygenation of distal tissue.

Brief Description of the Drawings

Figures 1A - 1C represent a schematic flow diagram of a method for producing a stable polymerized hemoglobin blood-substitute according to the present invention.

Figure 2 is a plot of arterial oxygen per gram of red blood cell hemoglobin for acutely normovolemic, hemodiluted, splenectomized beagle dogs, breathing room air and treated with varying amounts of hemoglobin blood-substitute or of a synthetic colloid solution (RHEOMACRODEX®-Saline) which is 10% Dextrane 40 and 0.9% saline.

Figure 3 is a plot of total arterial oxygen content for acutely normovolemic, hemodiluted, splenectomized beagle dogs, breathing room air and treated with varying amounts of hemoglobin blood-substitute or of a synthetic colloid solution (RHEOMACRODEX®-Saline).

Figure 4 is a plot of oxygen delivery for acutely normovolemic, hemodiluted, splenectomized beagle dogs, breathing room air and treated with varying amounts of hemoglobin blood-substitute or of a synthetic colloid solution (RHEOMACRODEX®-Saline).

Figure 5 is a plot of mean hind limb tissue oxygen tensions (in torr), for the experimental dogs described in Example 9, under the following conditions 1) baseline with a mean RBC hemoglobin (Hb) concentration of 15.8 g/dL, 2) after isovolemic hemodilution with hetastarch to a mean RBC hemoglobin concentration of 3.0 g/dL, 3) after isovolemic hemodilution with hetastarch, to a mean RBC hemoglobin concentration of 3.0 g/dL, and infusion of polymerized hemoglobin solution to achieve a plasma Hb

concentration increase of about 0.6 g/dL, resulting in a total hemoglobin concentration of about 3.6 g/dL, 4) after isovolemic hemodilution with hetastarch, to a mean RBC hemoglobin concentration of 3.0 g/dL, and infusion of polymerized hemoglobin solution to achieve a plasma Hb concentration increase of about 1.6 g/dL, resulting in a total hemoglobin concentration of 4.6 g/dL, and 5) after isovolemic hemodilution with hetastarch, to a mean RBC hemoglobin concentration of 3.0 g/dL, and infusion of polymerized hemoglobin solution to achieve a plasma Hb concentration increase of about 2.6 g/dL, resulting in a total hemoglobin concentration of 5.6 g/dL.

Figure 6 is a plot of mean hind limb tissue oxygen tensions (in torr), for the control dogs as compared to the Experimental Group A dogs, described in Example 10, for the following conditions 1) baseline, 2) 30 minutes after establishing a femoral artery stenosis in each dog (i.e. a 94% stenosis for the Experimental Group A dogs and a 90-93% stenosis for the Control Group dogs), 3) 30 minutes after intravenously injecting amounts of polymerized hemoglobin solution into the experimental dogs (or equivalent volumes of hetastarch solution into the control dogs), in the general circulatory system of each of the dogs proximal to the stenosis, in an amount sufficient to increase plasma hemoglobin concentration by about 0.5 grams per deciliter, and 4) 30 minutes after intravenously injecting amounts of polymerized hemoglobin solution into the experimental dogs (or equivalent volumes of hetastarch solution into the control dogs), in the general circulatory system of each of the dogs proximal to the stenosis, in an amount sufficient to increase plasma hemoglobin concentration by about 1.2 grams per deciliter.

Figure 7 is a plot of mean hind limb tissue oxygen tensions (in torr) for the Experimental Group B dogs,

described in Example 10, in which a 94% femoral artery stenosis was induced in a hind limb of each dogs after intravenously injecting amounts of polymerized hemoglobin solution into in the general circulatory system of each of the dogs proximal to the future stenosis, in an amount sufficient to increase total hemoglobin concentration by about 2 grams per deciliter. The plot provides mean hind limb tissue oxygen tensions for the following conditions 1) baseline, 2) 30 minutes after establishing a 94% femoral artery stenosis in each dog, and 3) 45 minutes after establishing a 94% femoral artery stenosis in each dog.

## Detailed Description of the Invention

The features and other details of the process of the invention will now be more particularly described with reference to the accompanying drawings and pointed out in the claims. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as limitations of the invention. The principle features of this invention can be employed in various embodiments without departing from the scope of the present invention.

As defined herein, a blood-substitute is a hemoglobin-based oxygen carrying composition for use in humans, mammals and other vertebrates, which is capable of transporting and transferring oxygen to vital organs and tissues, at least, and can maintain sufficient intravascular oncotic pressure. A vertebrate is as classically defined, including humans, or any other vertebrate animals which uses blood in a circulatory system to transfer oxygen to tissue. A preferred vertebrate for the method of invention is a mammal, such as a primate, a dog, a cat, a rat, a horse, a pig or a sheep. An even more preferred vertebrate is a human. A

vertebrate treated in the method of invention can be a fetus (prenatal vertebrate), a post-natal vertebrate, or a vertebrate at time of birth.

Additionally, the definition of circulatory system is as classically defined, consisting of the heart, arteries, veins and microcirculation including smaller vascular structures such as capillaries.

A blood-substitute formed by the method of invention must have levels of endotoxins, phospholipids, foreign proteins and other contaminants which will not result in a significant immune system response and which are non-toxic to the recipient. Preferably, a blood-substitute is ultrapure. Ultrapure as defined herein, means containing less than 0.5 EU/ml of endotoxin, less than 3.3 nmoles/ml phospholipids and no detectable levels of non-hemoglobin proteins, such as serum albumin or antibodies.

The term "endotoxin" refers to the cell-bound lipopolysaccharides, produced as a part of the outer layer of grain-negative bacterial cell walls, which under many conditions are toxic. When injected into animals, endotoxins can cause fever, diarrhea, hemorrhagic shock, and other tissue damage. Endotoxin unit (EU) has been defined by the United States Pharmacopeial Convention of 1983, page 3014, as the activity contained in 0.1 nanograms of U.S. reference standard lot EC-5. One vial of EC-5 contains 10,000 EU.
Examples of suitable means for determining endotoxin concentrations in a blood-substitute include the method "Kinetic/ Turbidimetric Limuus Amebocytic Lystate (LAL) 5000 Methodology" developed by Associates of Cape Cod, Woods Hole, Massachusetts.

Stable polymerized hemoglobin, as defined herein, is a hemoglobin-based oxygen carrying composition which does not substantially increase or decrease in molecular

weight distribution and/or in methemoglobin content during storage periods at suitable storage temperatures for periods of two years or more, and preferably for periods of two years or more, when stored in a low oxygen environment. Suitable storage temperatures for storage of one year or more are between about 0°C and about 40°C. The preferred storage temperature range is between about 4°C and about 30°C.

A suitable low oxygen environment is defined as the cumulative amount of oxygen in contact with the blood-substitute, over a storage period of at least one year which will result in a methemoglobin concentration of less than about 15% by weight in the blood-substitute. The cumulative amount of oxygen includes oxygen in leakage into the blood-substitute packaging and the original oxygen content of the blood-substitute and packaging.

Throughout this method, from RBC collection until hemoglobin polymerization, blood solution, RBCs and hemoglobin are maintained under conditions sufficient to minimize microbial growth, or bioburden, such as maintaining temperature at less than about 20°C and above 0°C. Preferably, temperature is maintained at a temperature of about 15°C or less. More preferably, the temperature is maintained at 10 ± 2°C.

In this method, portions of the components for the process for preparing stable polymerized hemoglobin solutions, and blood-substitutes derived therefrom, are sufficiently sanitized to produce a sterile product. Sterile is as defined in the art, specifically, that the solution meets United States Pharmacopeia requirements for sterility provided in *USP* XXII, Section 71, pages 1483-1488. Further, portions of components that are exposed to the process stream, are usually fabricated or clad with a material that will not react with or

contaminate the process stream. Such materials can include stainless steel and other steel alloys, such as Inconel.

One embodiment of the method, for producing a stable polymerized hemoglobin blood-substitute, system 10, is illustrated in Figures 1A - 1C. In Figure 1A, system 10 includes blood feed subsystem 12. Blood feed subsystem 12 includes strainer 14, feed tank 16, feed pump 18, and in-series prefilters 20,22. Feed tank 16 is filled with a blood solution comprising red blood cells (RBCs) or hemoglobin, and at least one anticoagulant.

Suitable RBC or hemoglobin sources, which can be processed in system 10, include new, old or outdated human blood, bovine blood, ovine blood, porcine blood, equine blood, and blood from other vertebrates and transgenically-produced hemoglobin, such as the transgenic Hb described in *BIO/TECHNOLOGY*, 12: 55-59 (1994); and recombinantly produced hemoglobin (*Nature*, *356*:258-60 (1992)).

The blood can be collected from live or freshly slaughtered donors. One method for collecting bovine whole blood is described in U.S. Patent Nos. 5,084,558 and 5,296,465, issued to Rausch *et al*. It is preferred that the blood be collected in a sanitary manner.

At or soon after collection, the blood is mixed with at least one anticoagulant to prevent significant clotting of the blood. Suitable anticoagulants for blood are as classically known in the art and include, for example, sodium citrate, ethylenediaminetetraacetic acid and heparin. When mixed with blood, the anticoagulant may be in a solid form, such as a powder, or in an aqueous solution.

It is understood that the blood solution source can be from a freshly collected sample or from an old sample, such as expired human blood from a blood bank. Further,

the blood solution could previously have been maintained in frozen and/or liquid state. It is preferred that the blood solution is not frozen prior to use in this method.

In another embodiment, prior to introducing the blood solution to Feed Tank 16, antibiotic levels in the blood solution, such as penicillin, are assayed. Antibiotic levels are determined to provide a degree of assurance that the blood sample is not burdened with an infecting organism by verifying that the donor of the blood sample was not being treated with an antibiotic. Examples of suitable assays for antibiotics include a penicillin assay kit (Difco, Detroit, MI) employing a method entitled "Rapid Detection of Penicillin in Milk". It is preferred that blood solutions contain a penicillin level of less than or equal to about 0.008 units/ml. Alternatively, a herd management program to monitor the lack of disease in or antibiotic treatment of the cattle may be used.

Preferably, the blood solution is strained prior to or during feed tank 16 filling to remove large aggregates and particles. A 600 mesh screen is an example of a suitable strainer.

Referring back to Figure 1A, feed pump 18 takes a suction on the blood solution in feed tank 16 and discharges the blood solution through in-series prefilters 20,22, to remove large blood solution debris of a diameter approximately 50 micrometers ("$\mu$m") or more, into diafiltration tank 24 in preparation for washing the RBCs contained in the blood solution. Examples of suitable in-series prefilters are 800 $\mu$m and 50 $\mu$m polypropylene filters. In the method of invention, prefiltration of the blood solution by in-series prefilters 20,22 is preferred to improve process efficiency. In another embodiment the blood solution is not prefiltered, rather large debris is removed by

subsequent ultrafiltration and/or chromatographic purification steps.

The RBCs in the blood solution are then washed by suitable means, such as by diafiltration or by a combination of discrete dilution and concentration steps with at least one isotonic solution, to separate RBCs from extracellular plasma proteins, such as serum albumins or antibodies (e.g., immunoglobulins (IgG)). It is understood that the RBCs can be washed in a batch or continuous feed mode.

Acceptable isotonic solutions are as known in the art and include solutions, such as a citrate/saline solution, having a pH and osmolarity which does not rupture the cell membranes of RBCs and which displaces the plasma portion of the whole blood. A preferred isotonic solution has a neutral pH and an osmolarity between about 285-315 mOsm. In a preferred embodiment, the isotonic solution is composed of an aqueous solution of sodium citrate dihydrate (6.0 g/l) and of sodium chloride (8.0 g/l).

Water as used in the method of invention may be distilled water, deionized water, water-for-injection (WFI) and/or low pyrogen water (LPW). WFI, which is preferred, is deionized, distilled water that meets U.S. Pharmacological Specifications for water-for-injection. WFI is further described in *Pharmaceutical Engineering*, 11, 15-23 (1991). LPW, which is preferred, is deionized water containing less than 0.002 EU/ml.

It is preferred that the isotonic solution is filtered prior to being added to the blood solution. Examples of suitable filters include a Millipore 10,000 Dalton ultrafiltration membrane, such as a Millipore Cat # CDUF 050 G1 filter or A/G Technology hollow fiber, 10,000 Dalton (Cat # UFD-10-C-85).

Referring back to Figure 1A, cell washing subsystem 26 contains diafiltration tank 24, diafiltration loop 28, discharge line 30, and pump 32. Suitable pumps include a Waukeshaw 100 gallon per minute Model W30. Pump 32 takes suction from diafiltration tank 24 and discharges to diafiltration loop 28 or discharge line 30. Diafiltration loop 28, which includes diafilter 34, recirculates flow from pump 32 to diafiltration tank 24. Temperature inside diafiltration tank 24 may be maintained by cooling the outside of diafiltration tank 24 using an ethylene glycol jacketed cooling system, not shown.

In a preferred embodiment, RBCs in the blood solution are washed by diafiltration. The blood solution contained in diafiltration tank 24 is circulated by pump 32, through diafiltration loop 28, whereby the blood solution is concentrated by the loss of filtrate across diafilter 34. Suitable diafilters include microporous membranes with pore sizes which will separate RBCs from substantially smaller blood solution components, such as a 0.1 μm to 0.5 μm filter (e.g., a 0.2 μm hollow fiber filter, Microgon Krosflo II microfiltration cartridge). Concurrently, filtered isotonic solution is added continuously (or in batches) as makeup at a rate equal to the rate (or volume) of filtrate lost across diafilter 34. During RBC washing, components of the blood solution which are significantly smaller in diameter than RBCs, or are fluids such as plasma, pass through the walls of diafilter 34 in the filtrate. RBCs, platelets and larger bodies of the diluted blood solution, such as white blood cells, are retained and mixed with isotonic solution, which is added continuously or batchwise to form a dialyzed blood solution.

In a more preferred embodiment, the volume of blood solution in diafiltration tank 24 is initially diluted by

the addition of a volume of a filtered isotonic solution to diafiltration tank 24. Preferably, the volume of isotonic solution added is about equal to the initial volume of the blood solution.

In an alternate embodiment, the RBCs are washed through a series of sequential (or reverse sequential) dilution and concentration steps, wherein the blood solution is diluted by adding at least one isotonic solution, and is concentrated by flowing across a filter, thereby forming a dialyzed blood solution.

RBC washing is complete when the level of plasma proteins contaminating the RBCs has been significantly reduced, typically by about 90%. Typically, RBC washing is complete when the volume of filtrate drained from diafilter 34 equals about 300%, or more, of the volume of blood solution contained in diafiltration tank 24 prior to diluting the blood solution with filtered isotonic solution. Referring back to Figure 1A, the volume of filtrate discharged through diafilter 34 is measured by a drain line flow meter, not shown. Additional RBC washing may further separate extracellular plasma proteins from the RBCs. For instance, diafiltration with 6 volumes of isotonic solution may remove at least about 99% of IgG from the blood solution.

The dialyzed blood solution is then exposed to means for separating the RBCs in the dialyzed blood solution from the white blood cells and platelets, such as by centrifugation. In Figure 1A, the dialyzed blood solution is continuously pumped by pump 32 from diafiltration tank 24 to a centrifuge 36, which is operating while concurrently being fed dialyzed blood solution by pump 32, to separate the RBCs from the white blood cells and platelets. During operation, centrifuge 36 rotates at a rate sufficient to separate the RBCs into a heavy RBC phase, while also separating a substantial

portion of the white blood cells (WBCs) and platelets into a light WBC phase. A fraction of the RBC phase and of the WBC phase are continuously discharged separately from centrifuge 36 during operation. Suitable centrifuges include, for example, a Sharples Super Centrifuge, Model # AS-16, fitted with a #28 ringdam.

It is understood that other methods known in the art for separating RBCs from other blood components can be employed, such as sedimentation, wherein the separation method does not rupture the cell membranes of a significant amount of the RBCs, such as less than about 30% of the RBCs, prior to RBC separation from the other blood components.

Following separation of the RBCs, the RBCs are lysed by a means for lysing RBCs to release hemoglobin from the RBCs to form a hemoglobin-containing solution. Lysis means can include various lysis methods, such as mechanical lysis, chemical lysis, hypotonic lysis or other known lysis methods which release hemoglobin without significantly damaging the ability of the Hb to transport and release oxygen.

Referring back to Figure 1A, it is preferred that a substantial portion of the RBCs contained in the RBC phase are mechanically lysed while discharging from centrifuge 36. The cell membranes of the RBCs rupture upon impacting a generally rigid surface or structure, such as the wall of RBC phase discharge line 38, thereby releasing hemoglobin (Hb) from the RBCs into the RBC phase. In a more preferred embodiment, the flow of the RBC phase through RBC phase discharge line 38 is at an angle to the flow of RBC phase out of centrifuge 36, thereby causing a substantial portion of the RBCs to be mechanically lysed by impacting the inner surface of discharge line 38.

In yet another embodiment, recombinantly produced hemoglobin, such as the recombinantly produced hemoglobin described in *Nature*, 356: 258-260 (1992), can be processed in the method of invention in place of RBCs. The bacteria cells containing the hemoglobin are washed and separated from contaminants as described above. These bacteria cells are then mechanically ruptured by means known in the art, such as a ball mill, to release hemoglobin from the cells and to form a lysed cell phase. This lysed cell phase is then processed as is the lysed RBC phase.

Following lysis, the lysed RBC phase is then ultrafiltered to remove larger cell debris, such as proteins with a molecular weight above about 100,000 Daltons. Generally, cell debris include all whole and fragmented cellular components with the exception of Hb, smaller cell proteins, electrolytes, coenzymes and organic metabolic intermediates. Acceptable ultrafilters include, for example, 100,000 Dalton filters made by Millipore (Cat # CDUF 050 H1) and made by A/G Technology (Needham, MA.; Model No. UFP100E55).

As shown in Figure 1A, the lysed RBC phase then flows into RBC phase discharge line 38 and is then pumped by pump 39 through clarification bypass line 40 into tank 42. The lysed RBC phase in tank 42 is then pumped by pump 44 through ultrafilter 46. A substantial portion of the Hb and water, contained in the lysed RBC phase, permeates ultrafilter 46 to form a Hb ultrafiltrate while larger cell debris, such as cell membranes and proteins with a molecular weight above about 100,000 Daltons, are retained and recirculated back into tank 42. Concurrently, water is continuously added to tank 42 as makeup for at least some of the fluid lost in the Hb ultrafiltrate.

It is preferred that ultrafiltration continues until the concentration of Hb in tank 42 is less than 8 grams/liter (g/l) to maximize the yield of hemoglobin available for polymerization. Other methods for separating Hb from the lysed RBC phase can be employed, including sedimentation, centrifugation or microfiltration. The temperature of tank 42 is controlled by suitable means, such as by cooling the outside of tank 42 through use of an ethylene glycol jacketed cooling system, not shown.

In an alternate embodiment, the lysed RBC phase flows through from RBC phase discharge line 38 through clarification line 47 into static mixer 48. Concurrent with the transfer of the RBC phase to static mixer 48, water is also injected into static mixer 48, preferably an approximately equal amount, by means not shown, wherein the water is then mixed under low shear conditions with the RBC phase to form a lysed RBC colloid.

It is understood that other means of low shear mixing, which are known in the art, can be used provided that the mixing means will not fragment a substantial amount of the RBC membranes, present in the lysed RBC phase, during mixing.

The lysed RBC colloid is then pumped from static mixer 48 by pump 50 into a suitable means to separate the Hb from non-hemoglobin RBC components, such as a sedimentation means or centrifuge 52 which is operated to separate the Hb from the RBC membrane, membrane associated proteins and other components. Centrifuge 52 rotates at a rate sufficient to separate the lysed RBC colloid into a light Hb phase and a heavy phase. The light phase is composed of Hb and typically contains non-hemoglobin components with a density approximately equal to or less than the density of Hb. An example of an

acceptable centrifuge is a Sharples Super Centrifuge, Model # AS-16 from Sharples Division of Alfa-Laval Separation, Inc. The Hb phase continuously discharges from centrifuge 52 during Hb separation and is collected in holding tank 54 in preparation for Hb purification. In another embodiment, pump 55 then transfers the Hb phase from holding tank 54 through microfilter 56, whereby the Hb in the Hb phase is cross-flow filtered from cell stroma yielding a Hb microfiltrate. Cell stroma are then returned with the retentate from microfilter 56 to holding tank 54 Suitable microfilters include a 0.45 μ Millipore Pellicon Cassette, Cat # HVLP 000 C5 microfilter. The temperature of holding tank 54 is controlled by suitable means, such as by cooling the outside of holding tank 54 through use of an ethylene glycol jacketed cooling system, not shown. To optimize performance of this method, when the fluid pressure at the inlet of microfilter 56 increases from an initial pressure of about 10 psi to about 25 psi, microfiltration is complete. Pump 58 then transfers the Hb microfiltrate from microfilter 56 into tank 42, after which the Hb microfiltrate is ultrafiltered by ultrafilter 46 to form a Hb ultrafiltrate.

The Hb ultrafiltrate is then ultrafiltered to remove smaller cell debris, such as electrolytes, coenzymes, metabolic intermediates and proteins less than about 30,000 Daltons in molecular weight, and water from the Hb ultrafiltrate. Suitable ultrafilters include a 30,000 Dalton ultrafilter (Millipore Cat # CDUF 050 T1 and/or Armicon, # 540 430).

Referring to Figure 1B, the Hb ultrafiltrate is pumped by pump 60 into ultrafiltrate tank 62. The Hb ultrafiltrate is then recirculated by pump 64 through ultrafilter 66, and back into ultrafiltration tank 62,

thereby forming a Hb retentate which is typically a concentrated Hb solution.

Preferably, ultrafiltration continues until the concentration of Hb in ultrafiltrate tank is equal to 100 g/l to improve the efficiency of subsequent purification steps.

The concentrated Hb solution is then directed into one or more parallel chromatographic columns to further separate the hemoglobin by high performance liquid chromatography from other contaminants such as antibodies, endotoxins, phospholipids, enzymes and viruses.

The concentrated Hb solution is then directed from ultrafiltrate tank 62 by pump 68 onto the medium contained in chromatographic columns 70 to separate the Hb from contaminants. Chromatographic column 70 must contain a volume of a medium which is suitable and sufficient to separate Hb from non-hemoglobin components of the ultrafiltrate. Examples of suitable media include anion exchange media, cation exchange media, hydrophobic interaction media and affinity media. In a preferred embodiment, chromatographic columns 70 contain an anion exchange medium suitable to separate Hb from non-hemoglobin proteins. Suitable anion exchange mediums include, for example, silica, alumina, titania gel, cross-linked dextran, agarose or a derivitized moiety, such as a polyacrylamide, a polyhydroxyethyl-methacrylate or a styrene divinylbenzene, that has been derivatized with a cationic chemical functionality, such as a diethylaminoethyl or quaternary aminoethyl group. A suitable anion exchange medium and corresponding eluants for the selective absorption and desorption of Hb as compared to other proteins and contaminants, which are likely to be in a lysed RBC phase, are readily determinable by one of reasonable skill in the art.

In a more preferred embodiment, a method is used to form an anion exchange media from silica gel, which is hydrothermally treated to increase the pore size, exposed to γ-glycidoxy propyltrimethoxysilane to form active epoxide groups and then exposed to dimethylaminoethanol ($HOCH_2CH_2N(CH_3)_2$) to form a quaternary ammonium anion exchange medium. This method is described in the *Journal of Chromatography*, 120:321-333 (1976), which is incorporated herein by reference in its entirety.

Chromatographic columns 70 are first pre-treated by flushing chromatographic columns 70 with a first eluant which facilitates Hb binding. Concentrated Hb solution is then injected onto the medium in columns 70. After injecting the concentrated Hb solution, chromatographic columns 70 are then successively washed with different eluants through chromatographic columns 70 to produce a separate, purified Hb eluate.

In a preferred embodiment, a pH gradient is used in chromatographic columns 70 to separate protein contaminants, such as the enzyme carbonic anhydrase, phospholipids, antibodies and endotoxins from the Hb. Each of a series of buffers having different pH values, are sequentially directed by suitable means, such as pump 74, through chromatographic columns 70 to create a pH gradient within the medium in chromatographic column 70. It is preferred that the buffers be filtered, such as with a 10,000 Dalton depyrogenation membrane. The buffers used to separate Hb should have a low ionic strength such that elution of Hb and non-hemoglobin contaminants is generally dependent upon pH and not significantly dependent upon ionic strength. Typically, buffers with an ionic concentration of about 50 mM, or less, have suitable low ionic strengths.

The first buffer transports the concentrated Hb solution into the medium in chromatographic columns 70 and facilitates binding of the Hb to the medium. The second buffer then adjusts the pH within chromatographic columns 70 to eluate contaminating non-hemoglobin components from chromatographic columns 70 while maintaining the Hb bound to the medium. The third buffer then eluates the Hb from chromatographic columns 70. The Hb eluate is then collected in tank 76. It is preferred that the Hb eluate be directed through a sterile filter, such as sterile filter 77 before further use. Suitable sterile filters include 0.22 μm filters, such as a Sartorius Sartobran pH Cat # 5232507 G1PH filter.

In a preferred embodiment, the first 3%-to-4% of the Hb eluate and the last 3%-to-4% of the Hb eluate are directed to waste to provide assurance of the purity of the Hb eluate.

Wherein chromatographic columns 70 are to be reused, contaminating non-hemoglobin proteins and endotoxin, remaining in chromatographic columns 70, are then eluted by a fourth buffer. The fourth buffer does not need to have a low ionic strength as the Hb has already been separated and eluated from chromatographic columns 70.

In a preferred embodiment, the first buffer is a tris-hydroxymethyl aminomethane (Tris) solution (concentration about 20mM; pH about 8.4 to about 9.4). The second buffer is a mixture of the first buffer and a third buffer, with the second buffer having a pH of about 8.2 to about 8.6. The third buffer is a Tris solution (concentration about 50 mM; pH about 6.5 to about 7.5). The fourth buffer is a NaCl/Tris solution (concentrations about 1.0 M NaCl and about 20 mM Tris; pH about 8.4 to about 9.4, preferably about 8.9-9.1). It is particularly preferred that the pH of the second buffer be between about 8.2 and about 8.4.

Typically, the buffers used are at a temperature between about 0°C and about 50°C. Preferably, buffer temperature is about 12.4 ± 1.0°C during use. In addition, the buffers are typically stored at a temperature of about 9°C to about 11°C.

The Hb eluate is then deoxygenated prior to polymerization to form a deoxygenated Hb solution (hereinafter deoxy-Hb) by means that substantially deoxygenate the Hb without significantly reducing the ability of the Hb in the Hb eluate to transport and release oxygen, such as would occur from denaturation or formation of oxidized hemoglobin (met Hb).

In one embodiment, the Hb eluate is deoxygenated by gas transfer of an inert gas across a phase transfer membrane. Such inert gases include, for example, nitrogen, argon and helium. It is understood that other means for deoxygenating a solution of hemoglobin, which are known in the art, can be used to deoxygenate the Hb eluate. Such other means, can include, for example, nitrogen purging of the Hb eluate; or photolysis by light; chemical scavenging with reducing agents such as sulfhydryl compounds such as N-acetyl-L-cysteine (NAC) D,L-cysteine, $\gamma$-glutamyl-cysteine, glutathione, 2-mercaptoethanol, 2,3-dimercapto-1-proponal, 1,4-butanedithiol, thioglycalate, dithioerythritol, dithiothreitol, and other biologically compatible sulfhydryl compounds; citrate salts; citric acid; reduced nicotinamide adenine dinucleotide ($NADH_2$); reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$); reduced flavin adenine dinucleotide ($FADH_2$); and other biologically compatible reducing agents.

Referring back to Figure 1B, deoxygenation is accomplished within Hb deoxygenation subsystem 78. Hb deoxygenation subsystem 78 includes tank 76, pump 80,

recirculation piping 82, phase transf r membrane 86, and discharge line 90. Pump 80 takes suction on the Hb eluate in tank 76 and discharges to either recirculation piping 82 or through discharge line 90. Hb eluate pumped by pump 80 from tank 76 through recirculation piping 82 then flows through ultrafilter 84 and/or phase transfer membrane 86, which are in parallel, and subsequently returns to tank 76. Following elution from chromatographic column 70, the Hb eluate is preferably concentrated to improve the efficiency of the process. The Hb eluate is recirculated through ultrafilter 84 to concentrate the Hb eluate to form a concentrated Hb solution. Suitable ultrafilters include, for example, 30,000 or less Dalton ultrafilters (e.g., Millipore Helicon, Cat # CDUF050G1, Amicon Cat # 540430). Typically, concentration of the Hb eluate is complete when the concentration of Hb is between about 100 to about 120 g/l. While concentrating the Hb eluate, the Hb eluate temperature is preferably maintained at approximately 8-12°C within tank 76. The temperature of tank 76 is controlled by suitable means, such as by cooling the outside of tank 76 through use of an ethylene glycol jacketed cooling system, not shown.

Buffer is then directed, by pump 88, into the Hb solution in tank 76, which is preferably concentrated, to adjust the ionic strength of the Hb solution to enhance Hb deoxygenation. It is preferred that the ionic strength be adjusted to greater than about 150 meq/l to reduce the oxygen affinity of the Hb in the Hb solution. Suitable buffers include buffers with a pH that will not result in significant denaturing of the Hb protein but will have an ionic strength sufficiently high to promote Hb deoxygenation. Examples of suitable buffers include saline solutions with a pH range of about 6.5 to about

8.9. A preferred buffer is an aqueous 1.0 M NaCl, 20 mM Tris solution with a pH of about 8.9.

Preferably, the resulting buffered Hb solution is then recirculated through ultrafilter 84, and returned to tank 76, to again concentrate the Hb solution to improve the efficiency of the process. In a preferred embodiment, concentration is complete when the concentration of Hb is about 100 g/l to about 120 g/l.

During deoxygenation the Hb solution is recirculated by pump 80 from tank 76, through phase transfer membrane 86, and then back to tank 76. Appropriate phase transfer membranes include, for example, a 0.05 μm polypropylene hollow fiber microfilter (e.g., Hoechst-Celanese Cat # 5PCM-107). Concurrently, a counterflow of an inert gas is passed across phase transfer membrane 86. Suitable inert gases include, for example, nitrogen, argon and helium. Gas exchange across phase transfer membrane 86 thereby strips oxygen out of the Hb solution.

Deoxygenation is continued until a low oxygen concentration is achieved which is suitable to limit significant methemoglobin formation and which results in a large proportion of the Hb molecules having a structural configuration that will result in a low oxygen affinity when the Hb is cross-linked, thereby enhancing the oxygen delivery capacity of the hemoglobin. Typically, deoxygenation continues until $HbO_2$ concentration is less than about 20%, and preferably less than about 10%.

During deoxygenation, the temperature of the Hb solution is typically maintained at a level that will balance the rate of deoxygenation against the rate of methemoglobin formation. Temperature is maintained to limit methemoglobin content to less than 20%. An optimum temperature will result in less than about 5%

methemoglobin content, and preferably less than about 2.5% methemoglobin content, while still deoxygenating the Hb solution. Typically, during deoxygenation the temperature of the Hb solution is maintained between about 19°C and about 31°C.

During deoxygenation, and subsequently throughout the remaining steps of the method of invention, the Hb is maintained in a low oxygen environment to minimize oxygen absorption by the Hb solution. Suitable low oxygen environments include, for example, those environments which would result in an oxygenated Hb (oxyhemoglobin or $HbO_2$) of less than about 20 in the Hb solution.

The deoxy-Hb is then mixed with a suitable deoxygenated storage buffer and cross-linking agent. It is understood that the storage buffer and the cross-linking agent can be added to the deoxy-Hb concurrently, or the cross-linking agent can be added to the deoxy-Hb after adding the storage buffer. it is preferred that the storage buffer and cross-linking agent be added to the deoxy-Hb sequentially.

In one embodiment, the storage buffer is filtered before mixing with the deoxy-Hb. A suitable filter for a storage buffer is capable of depyrogenating the buffer, typically a 10,000 Dalton, or less, filter or membrane. Examples of a suitable storage buffer filter include Millipore Helicon, Cat # CDUF050G1 or AG Technology Maxcell, Cat # UFP-10-C-75 depyrogenating filters having exclusion limits of 10,000 Daltons.

In one embodiment, the storage buffer also contains a buffer to stabilize pH in order to keep the ionic strength of the deoxy-Hb low enough not to significantly interfere with intermolecular cross-linking during polymerization. Typically, the ionic strength of the

oxidation-stabilized deoxy-Hb is less than about 50 mOsm before polymerization.

A suitable deoxygenated storage buffer contains an amount of a non-toxic reducing agent suitable to stabilize the deoxy-Hb solution against oxidation. Suitable reducing agents include sulfhydryl compounds such as N-acetyl-L-cysteine (NAC) D,L-cysteine, $\gamma$-glutamyl-cysteine, glutathione, 2-mercaptoethanol, 2,3-dimercapto-1-proponal, 1,4-butanedithiol, thioglycalate, dithioerythritol, dithiothreitol, and other biologically compatible sulfhydryl compounds; citrate salts; citric acid; reduced nicotinamide adenine dinucleotide ($NADH_2$); reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$); reduced flavin adenine dinucleotide ($FADH_2$); and other biologically compatible reducing agents. The oxygen content of a low oxygen content storage buffer must be low enough not to significantly reduce the concentration of reducing agent in the buffer and to limit oxyhemoglobin content in oxidation stabilized deoxy-Hb to about 20% or less. Typically, the storage buffer has a $pO_2$ of less than about 50 torr. The functions of the sulfhydryl compound include deoxygenating oxygenated Hb remaining in the deoxy-Hb, reducing methemoglobin remaining in the deoxy-Hb, maintaining the deoxy-Hb (and a final Hb blood-substitute product) in an oxidation-stabilized (i.e., deoxygenated) state at room temperature by oxygen scavenging, and facilitating optimal Hb polymerization to preferentially form modified tetrameric Hb. Modified tetrameric Hb, as defined herein, is tetrameric Hb which has been intramolecularly cross-linked to preclude significant dissociation of the Hb tetramers into Hb dimers.

In a preferred embodiment, the storage buffer should have a pH suitable to balance Hb polymerization and

methemoglobin formation, typically between about 7.6 and about 7.9.

In a preferred embodiment, the storage buffer contains approximately 25-35 mM sodium phosphate buffer (pH 7.7-7.8) and contains an amount of NAC such that the concentration of NAC in oxidation stabilized deoxy-Hb is between about 0.003% and about 0.3%, by weight. More preferably, the NAC concentration in the oxidation stabilized deoxy-Hb is between about 0.05% and about 0.2% by weight.

The pH and oxygen content of the storage buffer should be suitable to ensure that the oxidation-stabilized deoxy-Hb will have a pH suitable to balance Hb polymerization and methemoglobin formation, preferably from 7.6 to about 7.9 pH units, and an oxygen content of less than about 20% $HbO_2$ after stabilization, preferably $\leq$ 10% $HbO_2$, also limiting methemoglobin formation.

In a preferred embodiment, an amount of an N-acetyl cysteine (NAC) storage buffer is added to the deoxy-Hb such that, before polymerization, the oxidation-stabilized deoxy-Hb contains from about 0.003 % to about 0.3% NAC, by weight.

In a more preferred embodiment, an amount of an NAC storage buffer is added to the deoxy-Hb such that, before polymerization, the oxidation-stabilized deoxy-Hb contains from about 0.03 % to about 0.3% NAC by weight, or an equivalent mole percent for other sulfhydryl compounds.

In an even more preferred embodiment, the oxidation-stabilized deoxy-Hb contains between about 0.05% and about 0.2% by weight NAC before initiating polymerization.

Referring back to Figure 1B, the storage buffer is added to the deoxy-Hb in tank 76 by suitable means, such

as pump 88. Concurrently, the deoxy-Hb is diafiltered through recirculation from tank 76 by pump 80 through ultrafilter 84. Typically, pump 80 (e.g., Albin Sanitary Pump Model 325, Albin Pump, Inc., Atlanta, GA) is designed to allow pressurized flow through pump 80 while pump 80 is not operating. The storage buffer is added, continuously or batchwise, to tank 76 at a amount generally sufficient to make up for the fluid loss across ultrafilter 86 by maintaining the fluid in tank 76 at approximately a constant level. Preferably, equilibration continues until the volume of fluid lost through diafiltration across ultrafilter 86 is about three times the initial volume of the deoxy-Hb in tank 76. The volume of filtrate discharged from ultrafilter 84 can be measured by means known in the art, such as a drain line flow meter.

As shown in Figures 1B and 1C, the oxidation-stabilized deoxy-Hb is subsequently transferred from Hb deoxygenation subsystem 78 through discharge line 90 by pressurizing tank 76 with an inert gas, such as nitrogen.

In one embodiment, the oxidation-stabilized deoxy-Hb then flows from discharge line 90 through optional filter 92 into storage tank 94. Suitable filters include a 0.5 µm polypropylene prefilter (Pall Profile II, Cat # ABIY005Z7) and a 0.2 µm sterile microfilter (Gelman Supor). Storage tank 94 is maintained under a low oxygen environment, such as by purging and blanketing with an inert gas, such as nitrogen, prior to filling with oxidation-stabilized deoxy-Hb. After filling with oxidation-stabilized deoxy-Hb, storage tank 94 is blanketed with an inert gas, such as nitrogen, and sealed to minimize bacterial or oxygen contamination. Storage tank 94 is typically maintained at approximately room temperature. Subsequently, the oxidation-stabilized deoxy-Hb is transferred from storage tank 94, by

pressurizing storage tank 94 with an inert gas, such as nitrogen, to deoxygenation loop 96 of polymerization subsystem 98.

In another embodiment, the oxidation-stabilized deoxy-Hb then flows directly from discharge line 90 through storage bypass line 95 into deoxygenation loop 96 by pressurizing tank 76 with an inert gas, such as nitrogen.

Polymerization subsystem 98 includes deoxygenation loop 96, concentration loop 100, and polymerization reactor 102. In deoxygenation loop 96, pump 104 recirculates the oxidation-stabilized deoxy-Hb from polymerization reactor 102 through static mixer 106, and then through phase transfer membrane 108. Concentration loop 100 includes pump 110 which takes a suction from polymerization reactor 102 and recirculates the oxidation-stabilized deoxy-Hb through ultrafilter 112, or through in-parallel bypass shunt 114, and then through static mixer 116. An example of an acceptable ultrafilter is a 30,000 Dalton ultrafiltration membrane (e.g., Millipore Helicon CDUF050LT or Amicon S40Y30). Suitable phase transfer membranes include, for example, a 0.05 $\mu$m polypropylene microfilter (e.g., Hoechst-Celanese Corporation Cat # 5PCM-108, 80 sq.ft.).

Optionally, prior to transferring the oxidation-stabilized deoxy-Hb to polymerization subsystem 98, an appropriate amount of water is added to polymerization reactor 102. In one embodiment an appropriate amount of water is that amount which would result in a solution with a concentration of about 10 to about 100 g/l Hb when the oxidation-stabilized deoxy-Hb is added to polymerization reactor 102. Preferably, the water is oxygen-depleted.

The water is recirculated by pump 104, throughout polymerization subsystem 98, to support deoxygenation of

the water by flow through phase transfer membrane 108. A counterfl w of a pressurized inert gas, such as nitrogen, is directed across the opposite side of phase transfer membrane 108 to further deoxygenate the water.

After the $pO_2$ of the water in polymerization subsystem 98 is reduced to a level sufficient to limit $HbO_2$ content to about 20%, typically less than about 50 torr, polymerization reactor 102 is blanketed with an inert gas, such as nitrogen. The oxidation-stabilized deoxy-Hb is then transferred from the storage tank 94, by pressurizing storage tank 94 with an inert gas (e.g., nitrogen), into polymerization reactor 102 or by discharging directly from discharge line 90, through storage bypass line 95, into polymerization reactor 102, which is concurrently blanketed with an appropriate flow of an inert gas.

The temperature of the oxidation-stabilized deoxy-Hb solution in polymerization reactor 102 is raised to a temperature to optimize polymerization of the oxidation-stabilized deoxy-Hb when contacted with a cross-linking agent. Conditions for polymerizing hemoglobin include heating the polymerization reaction mixture in the polymerization reactor 102 to a temperature which is high enough to cause intramolecular Hb cross-linking and sufficiently low to prevent significant Hb denaturation. Typically the polymerization reaction mixture is heated to a temperature from about 25°C to about 45°C for approximately 2 to 6 hours. However, at least a portion of the polymerization reaction mixture will polymerize at temperatures below 25°C. Preferred polymerization conditions include heating said reaction mixture to about 40°C to about 44°C for approximately 4-6 hours. An example of an acceptable heat transfer means, not shown, for heating polymerization reactor 102 is a jacketed

heating system which is heated by directing hot ethylene glycol through the jacket.

Examples of suitable cross-linking agents include polyfunctional agents that will cross-link Hb proteins, such as glutaraldehyde, succindialdehyde, activated forms of polyoxyethylene and dextran, α-hydroxy aldehydes, such as glycolaldehyde, N-maleimido-6-aminocaproyl-(2'-nitro,4'-sulfonic acid)-phenyl ester, m-maleimidobenzoic acid-N-hydroxysuccinimide ester, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, m-maleimidobenzoyl-N-hydroxysuccinimide ester, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester, N-succinimidyl(4-iodoacetyl)aminobenzoate, sulfosuccinimidyl(4-iodoacetyl)aminobenzoate, succinimidyl 4-(p-maleimidophenyl)butyrate, sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, N,N'-phenylene dimaleimide, and compounds belonging to the bis-imidate class, the acyl diazide class or the aryl dihalide class, among others.

A suitable amount of a cross-linking agent is that amount which will permit intramolecular cross-linking to stabilize the Hb and also intermolecular cross-linking to form polymers of Hb, to thereby increase intravascular retention. Typically, a suitable amount of a cross-linking agent is that amount wherein the molar ratio of cross-linking agent to Hb is in excess of about 2:1. Preferably, the molar ratio of cross-linking agent to Hb is between about 20:1 to 40:1.

Preferably, the polymerization is performed in a buffer with a pH between about 7.6 to about 7.9, having a chloride concentration less than or equal to about 35 mmolar.

Referring back to Figure 1C, a suitable amount of the cross-linking agent is added to the oxidation-stabilized deoxy-Hb through an orifice, not shown, while recirculating the oxidation-stabilized deoxy-Hb from polymerization reactor 102 through bypass shunt 114. The cross-linking agent and the oxidation-stabilized deoxy-Hb are then mixed by a means for mixing with low shear. A suitable low-shear mixing means includes static mixer 116. A suitable static mixer is, for example, a "Kenics" static mixer obtained from Chemineer, Inc.

In one embodiment, recirculating the oxidation-stabilized deoxy-Hb and the cross-linking agent through static mixer 116 causes turbulent flow conditions with generally uniform mixing of the cross-linking agent with the oxidation-stabilized deoxy-Hb thereby reducing the potential for forming pockets of deoxy-Hb containing high concentrations of the cross-linking agent. Generally uniform mixing of the cross-linking agent and the deoxy-Hb reduces the formation of high molecular weight Hb polymers, i.e. polymers weighing more than 500,000 Daltons, and also permits faster mixing of the cross-linking agent and the deoxy-Hb during polymerization.

Furthermore, if the reducing agent is a sulfhydryl compound, significant Hb intramolecular cross-linking will result during Hb polymerization due to the presence of the sulfhydryl compound, preferably NAC. While the exact mechanism of the interaction of the sulfhydryl compound with the cross-linking agent and/or Hb is not known, it is presumed that the sulfhydryl compound affects Hb/cross-linking agent chemical bonding in a manner that at least partially inhibits the formation of high molecular weight Hb polymers and preferentially forms stabilized tetrameric Hb.

The amount of a sulfhydryl compound mixed with the deoxy-Hb is an amount high enough to increase

intramolecular cross-linking of Hb during polymerization and low enough not to significantly decrease intermolecular cross-linking of Hb molecules, due to a high ionic strength. Typically, about one mole of sulfhydryl functional groups (-SH) are needed to oxidation stabilize between about 0.25 moles to about 5 moles of deoxy-Hb. If the reducing agent is not a sulfhydryl compound, a sulfhydryl compound is added to the polymerization reaction mixture in an amount sufficient to enhance intramolecular cross-linking. Appropriate sulfhydryl compounds include those compounds listed above. The sufficient amount of the sulfhydryl compound will vary; generally, between about 0.003% and 0.3%.

Poly(Hb) is defined as having significant intramolecular cross-linking if a substantial portion of the Hb molecules are chemically bound in the poly(Hb), and only a small amount, such as less than 15%, are contained within high molecular weight polymerized hemoglobin chains. High molecular weight poly(Hb) molecules are molecules, for example, with a molecular weight above about 500,000 Daltons.

In a preferred embodiment, glutaraldehyde is used as the cross-linking agent. Typically, about 10 to about 70 grams of glutaraldehyde are used per kilogram of oxidation-stabilized deoxy-Hb. More preferably, glutaraldehyde is added over a period of five hours until approximately 29-31 grams of glutaraldehyde are added for each kilogram of oxidation-stabilized deoxy-Hb. It is also preferred that polymerization is conducted in a buffer having a chloride ion concentration of less than or equal to about 35 mM. An example of a suitable buffer is a 12 mM phosphate buffer with a pH of 7.8.

After polymerization, the temperature of the poly(Hb) solution in polymerization reactor 102 is

typically reduced to a temperature that will inhibit any significant amount of further reactions with the cross-linking agent, about 15°C to about 25°C, and preferably, between about 18°C to about 20°C. An example of an acceptable heat transfer means for cooling polymerization reactor 102 is a polymerization reactor jacketed by a heat exchanger which is cooled by ethylene glycol flow, not shown.

Wherein the cross-linking agent used is not an aldehyde, the poly(Hb) formed is a stable poly(Hb). Wherein the cross-linking agent used is an aldehyde, the poly(Hb) formed is not stable until mixed with a suitable reducing agent to reduce less stable bonds in the poly(Hb) to form more stable bonds. Examples of suitable reducing agents include sodium borohydride, sodium cyanoborohydride, sodium dithionite, trimethylamine, t-butylamine, morpholine borane and pyridine borane.

Prior to adding a reducing agent, the poly(Hb) solution is optionally concentrated by recirculating the poly(Hb) solution through ultrafilter 112 until the concentration of the poly(Hb) solution is increased to between about 75 and about 85 g/l. An example of a suitable ultrafilter is a 30,000 Dalton filter (e.g., Millipore Helicon, Cat # CDUF050LT Amicon 540430).

If the reducing agent is borohybride, the pH of the poly(Hb) solution is then adjusted to the alkaline pH range to preserve the reducing agent and to prevent hydrogen gas formation, which can denature Hb during the subsequent imine reduction. In one embodiment, the pH is adjusted to greater than 10. The pH is adjusted by adding a buffer solution to the poly(Hb) solution in polymerization reactor 102 while concurrently diafiltering the poly(Hb) solution. The poly(Hb) solution is diafiltered by recirculating the poly(Hb) solution from polymerization reactor 102, by pump 110,

through ultrafilter 112. An example of a suitable filter for the sodium borate buffer is a 10,000 Dalton ultrafiltration membrane. Alternatively, the pH may be adjusted by direct addition of an alkaline buffer.

Following pH adjustment, at least one reducing agent, preferably a sodium borohydride solution, is added to polymerization reactor 102 typically through deoxygenation loop 96. Typically, about 5 to about 18 moles of reducing agent are added per mole of Hb tetramer (per 64,000 Daltons of Hb) within the poly(Hb). In a preferred embodiment, for every nine liters of poly(Hb) solution in polymerization subsystem 98, one liter of 0.25 M sodium borohydride solution is added at a rate of 0.1 to 0.12 lpm.

The amount of reducing agent, as a liquid, a solution and/or a slurry, is added to polymerization reactor 102 through an orifice, not shown, and then circulated through static mixer 106 and phase membrane 108, to result in turbulent flow conditions that support rapid, effective mixing of the reducing agent with the poly(Hb) solution in polymerization reactor 102. During the addition of the reducing agent, the poly(Hb) solution in polymerization reactor 102 is continuously recirculated by pump 104 through static mixer 106 and phase transfer membrane 108 to remove dissolved oxygen and hydrogen. After completion of the addition of the reducing agent, reduction continues in polymerization reactor 102 while an agitator contained therein, not shown, continues to mix the poly(Hb) solution and reducing agent for about 0.5 to 2 hours to form a stable poly(Hb).

Stable poly(Hb) in polymerization subsystem 98 is then concentrated, if necessary, to a concentration, typically between about 10 g Hb/l and about 250 g Hb/l. The stable poly(Hb) solution is concentrated by

recirculation from polymerization reactor 102, by pump 110, through ultrafilter 112.

The pH and electrolytes of the stable poly(Hb) are then restored to physiologic levels to form a stable polymerized hemoglobin blood-substitute, by diafiltering the stable poly(Hb) with a diafiltration solution having a suitable pH and physiologic electrolyte levels. Preferably, the diafiltration solution is a buffer solution.

Wherein the poly(Hb) was reduced by a reducing agent, the diafiltration solution has an acidic pH, preferably between about 4 to about 6.

A non-toxic reducing agent is also added to the stable poly(Hb) solution as an oxygen scavenger to enhance the stability of the final polymerized hemoglobin blood-substitute. The reducing agent can be added as part of the diafiltration solution and/or can be added separately. An amount of reducing agent is added to establish a concentration which will scavenge oxygen to maintain methemoglobin content less than about 15% over the storage period. Preferably, the reducing agent is a sulfhydryl compound, and more preferably is NAC. Typically, the amount of reducing agent added is an amount sufficient to establish a sulfhydryl concentration between about 0.05% and about 0.2% by weight.

Diafiltration continues until the exchange of volumes has been sufficient to establish physiologically acceptable pH and electrolyte levels in the stable poly(Hb) solution, thereby forming a stable polymerized hemoglobin blood-substitute. In Figure 1C, the volume of fluid typically lost through diafiltration across ultrafilter 112 is between about 6 to about 10 times the volume of the stable poly(Hb) in polymerization subsystem 98.

In another embodiment, after forming a stable polymerized hemoglobin blood-substitute, the blood-substitute is diafiltered to remove unmodified and modified tetrameric Hb from the blood-substitute. The blood-substitute is diluted, typically to a concentration of about 30 g/l to about 50 g/l, with the physiologic solution. The diluted Hb iq then diafiltered by recirculation through polymerization reactor 102, by pump 104 through static mixer 106 and purification filter 120. The diluted blood-substitute is then diafiltered with a physiologic solution, generally a deoxygenated buffer having physiologically acceptable pH and electrolyte levels, to reduce the content of modified tetrameric and unmodified tetrameric Hb species to about 10% or less, as determined by gel permeation chromatography when run under dissociating conditions. Modified tetrameric Hb is defined as tetrameric Hb which has been intramolecularly cross-linked to preclude significant dissociation of the Hb tetramers into Hb dimers. Purification filter 120 has a preferred molecular weight cutoff sufficient to remove modified and unmodified Hb tetramer from the blood-substitute, typically about 100,000 Daltons, Filtron Ex. Omega, Cat # 05100C05. Following removal of the modified and unmodified tetrameric Hb, recirculation of the blood-substitute is continued, if necessary, through ultrafilter 112 until the concentration of the Hb product is between about 10 g Hb/l and about 250 g Hb/l, and preferably between about 120 g Hb/l and about 140 g Hb/l.

In a preferred embodiment, the blood substitute is packaged under aseptic handling conditions while maintaining pressure with an inert, substantially oxygen-free atmosphere, in the polymerization reactor and remaining transport apparatus.

The specifications for a suitable stable polymerized hemoglobin blood-substitute formed by the method f inv ntion are provided in Table I.

Table I

| PARAMETER | RESULTS |
|---|---|
| pH (18-22°C) | Physiologically acceptable |
| Endotoxin | Physiologically acceptable |
| Sterility Test | Meets Test |
| Phospholipids[a] | Physiologically acceptable |
| Total Hemoglobin<br>Methemoglobin<br>Oxyhemoglobin | 10 - 250 g/l<br><15%<br>≤10% |
| Sodium, $Na^+$<br>Potassium, $K^+$<br>Chloride, $Cl^-$<br>Calcium, $Ca^{++}$<br>Boron | Physiologically acceptable |
| Glutaraldehyde | Physiologically acceptable |
| N-acetyl-L-cysteine | Physiologically Acceptable |
| M.W. >500,000 | ≤15% |
| M.W. ≤ 65,000 | ≤10% |
| M.W. ≤32,000 | ≤5% |
| Particulate Content ≥10μ | <12/ml |
| Particulate Content ≥25μ | <2/ml |

a - measured in Hb before polymerization

An example of a physiologic pH is pH between about 7.2 and about 7.9 at 18-22°C. A preferred physiologic pH is between about 7.6 and about 7.9 at 18-22°C.

Referring back to Figure 1C, polymerization reactor 102 is subsequently pressurized with an inert gas to transport the stable polymerized hemoglobin blood-substitute, through aseptic handling procedures, into a storage container. Optionally, the blood-substitute is directed through a prefilter and microfilter, not shown, prior to storage. A 0.5 μm or less polyproylene prefilter and a 0.2 μm sterile filter are acceptable as prefilters and microfilters, respectively.

The stable blood-substitute is then stored in a short-term storage container, not shown, or into sterile storage containers, each having a low oxygen environment. The storage container must also be sufficiently impermeable to water vapor passage to prevent significant concentration of the blood-substitute by evaporation over the storage period. Significant concentration of the blood-substitute is concentration resulting in one or more parameters of the blood-substitute being high out of specification.

In a preferred embodiment of the invention, stability of a hemoglobin blood substitute is preserved by maintaining the hemoglobin blood substitute in an atmosphere substantially free of oxygen. This method can be accomplished by maintaining the blood substitute in an oxygen-impermeable container, such as, an oxygen barrier film overwrap (e.g., a bag), glass container (e.g., a vial) or a steel container. Where the container is an oxygen barrier overwrap, the container can be manufactured from a variety of materials, including polymer films, (e.g., an essentially oxygen-impermeable polyester, ethylene vinal laminate (EVOH), polyvinylidine

dichlorine (PVDC) or nylon) and laminates, such as a foil laminate (e.g., a silver or aluminum foil laminate).

Where the overwrap is a film, such as a polyester film, the film can be rendered essentially oxygen-impermeable by a variety of suitable methods. In one embodiment, the film as manufactured is essentially oxygen-impermeable. Alternatively, where the polymeric material is not sufficiently oxygen-impermeable to meet the desired specifications, the film can be laminated or otherwise treated to reduce or eliminate the oxygen permeability.

In a preferred embodiment, a foil laminate is employed where the foil is an aluminum, silver, gold or other metal. The foil layer preferably has a thickness between about 0.0001 and 0.01 inches, more preferably about 0.003 inches. The laminate typically contains one or more polymeric layers. The polymer can be a variety of polymeric materials including, for example, a polyester layer (e.g., a 48 gauge polyester), polypropylene, nylon, etc.

The containers of the invention can be of a variety of constructions, including vials, cylinders, boxes, etc. In a preferred embodiment, the container is in the form of a bag. A suitable bag can be formed by continuously bonding one or more (e.g., two) sheets at the perimeter(s) thereof to form a tightly closed, oxygen impermeable, construction having a fillable center. The shape of the bag can be those routinely encountered in that art. The bonding can be achieved with any suitable material. In the case of a polyester/foil laminate material, a polyester adhesive can be employed for example.

The containers preferably have an oxygen permeability of less than ab ut 1.0 cc per 100 square inches per 24 hours per atmosphere at room temperature,

preferably less than about 0.6 cc per square inch at these conditions. Containers that meet these criteria include plastic containers with an overwrap, such as Cryovac products, such as Cryovac BYV200, and KAPAK products, such as KAPAK 50303 (KAPAK Corporation, Minneapolis, MN). These metal or polymeric composite film overwrapped plastic bags are sealed using an AUDIONVAC sealing apparatus (Audion Electro B.V., Weesp-Holland). KAPAK 50303 is a foil laminate container constructed from a 48 gauge polyester/foil/Sclair laminate. The foil layer is an aluminum foil with a thickness of about 0.0003". The Sclair layer (a polyethylene film) has a thickness of 0.003". Each layer is bonded by an adhesive. Other suitable KAPAK products include KAPAK 50703, KAPAK 50353 and KAPAK 60N32. Cryovac BYV200 is also a laminate product with a 0.6 mm layer of low density polyethylene (LLDPE), 0.6 mm two-sided Saran-coated polyvinyl alcohol and 2.2 mm LLDPE sealant. The oxygen permeability is about 0.02 cc/100 sq. in./24 hrs/atm/72°F/0% humidity and about 0.4 cc/100 sq. in./24 hrs/atm/72°F/100% humidity.

In a preferred embodiment, the blood substitute is packaged under an atmosphere which is substantially free of oxygen. Examples of suitable atmospheres include nitrogen, argon and helium.

Pumps used in the method of invention include peristaltic-type, diaphragm-type, gear-type, piston-type and rotary-lobe type pumps. Diaphragm-type pumps are available from Bran & Luebbe Inc., Buffalo Grove, Illinois. Suitable rotary-lobe pumps include the Albin SLP 110 P51 B1 sanitary lobe-rotary pump from Albin Pump Inc., Atlanta, GA. Rotary-lobe pumps can also be obtained from Waukesha Pumps, Waukesha, Wisconsin, or G & H Corporation, Kenosha, Wisconsin.

The synthesis of a stable polymerized hemoglobin blood-substitute, form d according to the method of invention, is further described in Example 1.

Stable polymerized hemoglobin blood-substitute compositions of this invention, which are useful in mammals or humans, are described in Tables IV and V of Example 3. In addition, the veterinary use of a Hb blood-substitute of this invention in canines is described in Examples 5-6. Further, the use of a stable polymerized hemoglobin blood-substitute of this invention in humans without any significant side effects, is described in Examples 7-8.

The hemoglobin solutions of the invention can be introduced into the circulatory system, to increase tissue oxygenation of tissue affected by a reduction in red blood cell (RBC) flow to the tissue. A reduction in RBC flow can result from a partial obstruction of RBC flow, from a reduction in the population of blood vessels associated with a tissue region, and/or from a cardiogenic dysfunction.

Oxygen transfer through a capillary to its associated tissue is typically characterized in terms of oxygen flux, which is defined as the mass of oxygen transported through the capillary per unit time. Classically, oxygen flux has been primarily associated with red blood cell flux, as RBCs normally carry 98% of the oxygen in arterial blood. Thus, when RBC flow through a capillary is significantly reduced, oxygen flux is reduced, thereby resulting in less oxygen transfer to the associated tissue, and possibly tissue hypoxia or tissue anoxia.

The method of this invention utilizes the capacity of hemoglobin, separate from RBCs, to carry oxygen within the plasma phase of the circulatory system and to transfer oxygen to tissue. Thus, for a vertebrate who

has been administered hemoglobin by introducing the hemoglobin into the circulatory system of the vertebrate, oxygen flux also depends on the increase in oxygen transferred by the administered hemoglobin when circulated through the vertebrate's circulatory system.

The oxygen transfer capacity of hemoglobin, circulated in the circulatory system, is demonstrated in Example 9, wherein anemic hypoxia within muscle tissue, as defined by a measured reduction in the tissue oxygen tension, which was induced by isovolemic hemodilution with hetastarch, was effectively treated by intravascularly administering small doses of a hemoglobin solution to the test subjects.

In the method of invention, tissue oxygenation at least partially occurs as a result of the transfer of oxygen from hemoglobin, circulated in the plasma phase of the circulatory system, to a tissue of a vertebrate. The tissue being oxygenated can be a small localized tissue area; a regionalized tissue area, such as a limb or organ; and/or tissue throughout the body of the vertebrate. Tissue with a reduced oxygen supply, resulting from reduced RBC flow to the affected tissue, can become hypoxic, as measured by a reduction in tissue oxygen tension, and even anoxic under extreme conditions, such as a prolonged complete restriction in oxygen supply.

Tissue hypoxia is a decrease in the oxygen tension (partial pressure of oxygen) below normal levels within the tissue. Tissue anoxia is a condition with no measurable oxygen partial pressure within the tissue.

Tissue oxygenation, which is measured in terms of oxygen tension (oxygen partial pressure) within the tissue, is determined as described in Example 9.

A vertebrate, having a localized, regional or systemic reduction in RBC flow, can have oxygen transport

systems which are otherwise normal, or can have additional abnormalities which can deleteriously affect oxygen transport and transfer in a portion of the body, or throughout the body as a whole.

In addition, in this method the vertebrate has a normovolemic blood volume prior to administration of the hemoglobin. A normovolemic blood volume is defined as a volume of blood within the circulatory system of the vertebrate which will not result in hypovolemic shock, such as can result from a major hemorrhage or a large loss of fluid secondary to vomiting, diarrhea, burns or dehydration. Typically, a normovolemic blood volume includes at least about 90% of the normal volume of blood for that vertebrate. In some cases a normovolemic volume can contain as little as about 80% of the normal blood volume without resulting in hypovolemic shock.

Furthermore, the blood constituting the normovolemic blood volume, contains at least about a normal concentration of RBCs. For example, the blood in a normovolemic blood volume of a human typically has a major vessel hematocrit of at least about 30%.

In this method, a vertebrate also has a normal, or higher than normal, systemic vascular resistance in the circulatory system, prior to administering the hemoglobin. A normal systemic vascular resistance is a vascular resistance which would not result in distributive shock, such as septic shock, in the vertebrate.

Reduced red blood cell flow includes any reduction in RBC flow, either localized, regionalized and/or systemic, below normal RBC flow levels, including a "no RBC flow" condition. Localized RBC flow consists of RBC flow through one or more capillaries within a capillary bed, wherein said capillaries would normally provide RBC flow to oxygenate a localized tissue area. Regionalized

RBC flow provides RBC flow to oxygenate a larger tissue area, such as a limb or organ. Systemic RBC flow is flow through the major circulatory systems of the body, thus providing RBCs to oxygenate the body as a whole.

In one embodiment of the method of invention, hemoglobin is administered to a vertebrate who has, or will have, a partial obstruction of the circulatory system, such as a stenosis or vascular blockage, in an amount that reduces or precludes RBC flow past the partial obstruction, but by which at least some plasma can flow. Administering hemoglobin increases tissue oxygenation in tissue distal to a localized or regionalized partial obstruction, and/or to increases tissue oxygenation throughout the body to treat a systemic partial obstruction.

In this method, the partial obstruction has at least one opening through which a plasma component, such as molecular hemoglobin, can flow to the affected tissue, wherein the plasma component has a molecular weight of about 16,000 Daltons or more. Preferably, the partial obstruction has at least one opening through which plasma components, with a molecular weight of about 32,000 Daltons or more (e.g., dimeric Hb) can flow to the affected tissue. More preferably, plasma components, having a molecular weight of about 64,000 Daltons or more, such as intramolecularly cross-linked tetrameric Hb, can flow past the partial obstruction to the affected tissue.

RBCs are significantly larger than hemoglobin, typically being 7-10 microns in diameter, therefore requiring significantly larger vascular openings, than does hemoglobin (40-100 nm in diameter), to flow past a partial obstruction.

Partial obstructions can occur at all tissue locations and in all blood vessels, such as arteries,

veins and capillaries. In addition, valves within the circulatory system, such as aortic, mitral and tricuspid valves, can also be partially obstructed. Further, chamber or sections of the heart can be partially obstructed, such as ventricular outflows and the ventricular opening to the pulmonary artery.

A partial obstruction of the circulatory system can be temporary, permanent or recurrent. A circulatory system partial obstruction can be caused by various means, such as vessel wall defects, disease, injury, aggregation of blood components, neoplasms, space-occupying lesions, infections, foreign bodies, compression, drugs, mechanical devices, vasoconstriction and vasospasms.

A stenosis of the circulatory system, as defined herein, is a narrowing of any canal, or lumen, in the circulatory system. Typically, a stenosis can result from disease, such as atherosclerosis; a vessel wall abnormality, such as a suture line from an arterial graft, a junction point of attachment for a graft or stent, a kink or deformity in a vessel, graft or stent, healed or scarred tissue from an injury or invasive procedure (e.g., catheterization, angioplasty, vascular stenting, vascular grafting with prosthesis, allogenic tissue and/or autologous tissue); a vascular prosthesis such as an artificial valve or vessel; compression, such as by a neoplastic mass, hematoma or mechanical means (e.g., clamp, tourniquet or cuff device); chemical poisoning or drug side effects; vasoconstriction; and vasospasms.

Examples of stenosis within valves or sections of the heart include aortic stenosis, buttonhole stenosis, calcific nodular stenosis, coronary ostial stenosis, double aortic stenosis, fish-mouth mitral stenosis, idiopathic hypertrophic subaortic stenosis, infundibular

stenosis, mitral stenosis, muscular subaortic stenosis, pulmonary stenosis, subaortic stenosis, subvalvar stenosis, supravalvar stenosis, tricuspid stenosis.

Vascular blockage is defined herein as a blockage within a canal or lumen of the circulatory system. Typical examples of blockages within a canal or lumen include in situ or embolized atheromatous material or plaques, aggregations of blood components, such as platelets, fibrin and/or other cellular components, in clots resulting from disease or injury or at the site of wound healing. Clots include thrombosis, embolisms and in an extreme case, abnormal coagulation states.

Other vascular blockages include blockages resulting from an infection by a microorganism or macroorganism within the circulatory system, such as fungal or heartworm infections.

Further, vascular blockages can result from foreign bodies contained within any canal or lumen in the circulatory system, such as a "GELFOAM®" absorbable gelatin sterile sponge for blocking blood flow during an invasive medical procedure, or a broken catheter tip.

In another embodiment of the method of invention, hemoglobin is administered to a vertebrate who has, or will have, a reduction in the population of functioning blood vessels supplying RBCs to a tissue area, with a consequential reduction in RBC flow to the affected tissue, whereby the administered hemoglobin increases tissue oxygenation for the affected tissue. A reduction in the population of blood vessels typically is the result of a burn (thermal, chemical or radiation) or of an invasive medical procedure, such as removing or cauterizing blood vessels.

In yet another embodiment of the method of invention, hemoglobin is administered to a vertebrate who has reduced systemic blood flow, and thus reduced RBC

flow, due to a cardiogenic dysfunction, whereby the administered hemoglobin increases tissue oxygenation for tissue throughout the body. Cardiogenic dysfunctions are diseases, or injuries, of the heart, or affecting the heart, which result in low blood flow conditions, such as myocardial infarction, myocardial ischemia, myocardial injury, arrhythmia, cardiomyopathy, cardioneuropathy and pericardial effusion.

In this method, a partial obstruction of the circulatory system of a prenatal vertebrate is typically the result of a disease or defect affecting prenatal development during gestation, or from treatment of a disease or defect (e.g., in-utero surgery).

The improvement in oxygen transfer to tissue affected by reduced RBC flow, by intravascular administration of a hemoglobin solution, is demonstrated by the significant increases in tissue oxygenation, observed in Examples 9 and 10, following the intravascular infusion of sufficient doses of a hemoglobin solution to restore tissue oxygen tensions to baseline values.

The hemoglobin, when used in this method of invention, is not contained in a natural RBC, but rather, is typically present in a physiologically acceptable carrier. It is preferred that the carrier be in a liquid state. It is also preferred that the hemoglobin is present within a physiologically acceptable solution or suspension of hemoglobin within a physiologically acceptable carrier. Suitable hemoglobins include the hemoglobin solutions described herein, or other forms of hemoglobin, such as dimeric hemoglobin, tetrameric hemoglobin, intramolecularly cross-linked hemoglobin, polymerized hemoglobin, freeze-dried hemoglobin, and/or chemically modified hemoglobin, wherein a significant portion of the hemoglobin is capable of transporting and

transferring oxygen. Hemoglobin has a significant capability to transport and transfer oxygen if administration of the hemoglobin, into the circulatory system of a vertebrate, results in a measurable increase in tissue oxygen tension for hypoxic tissue in the body of the vertebrate. Preferably, at least about 85% of the hemoglobin is capable of transporting and transferring oxygen.

Examples of other suitable hemoglobin solutions include hemoglobin solutions which have a stabilized 2,3-diphosphoglycerate level, as described in U.S. Patent No. 3,864,478, issued to Bonhard; cross-linked hemoglobin, as described in U.S. Patent No. 3,925,344, issued to Mazur, or in U.S. Patent Nos. 4,001,200, 4,001,401 and 4,053,590, issued to Bonsen *et al.*, or in U.S. Patent No. 4,061,736, issued to Morris *et al.*, or in U.S. Patent No. 4,473,496, issued to Scannon; stroma-free hemoglobin, as described in U.S. Patent No. 3,991,181, issued to Doczi, or in U.S. Patent No. 4,401,652, issued to Simmonds *et al.* or in U.S. Patent No. 4,526,715, issued to Kothe *et al.*; hemoglobin coupled with a polysaccharide, as described in U.S. Patent No. 4,064,118, issued to Wong; hemoglobin condensed with pyridoxal phosphate, as described in U.S. Patent No. 4,136,093, issued to Bonhard *et al.*; dialdehyde-coupled hemoglobin, as described in U.S. Patent No. 4,336,248, issued to Bonhard *et al.*; hemoglobin covalently bound with inulin, as described in U.S. Patent No. 4,377,512, issued to Ajisaka *et al.*; hemoglobin or a hemoglobin derivative which is coupled with a polyalkylene glycol or a polyalkylene oxide, as described in U.S. Patent No. 4,412,989, issued to Iwashita *et al.*, or U.S. Patent No. 4,670,417, issued to Iwasaki *et al.*, or U.S. Patent No. 5,234,903, issued to Nho *et al.*; pyrogen- and stroma-free hemoglobin solution, as described in U.S. Patent No. 4,439,357, issued to

Bonhard *et al.*; stroma-free, non-heme protein-free hemoglobin, as described in U.S. Patent No. 4,473,494, issued to Tye; modified cross-linked stroma-free hemoglobin, as described in U.S. Patent No. 4,529,719, issued to Tye; stroma-free, cross-linked hemoglobin, as described in U.S. Patent No. 4,584,130, issued to Bucci *et al.*; α-cross-linked hemoglobin, as described in U.S. Patent Nos. 4,598,064 and Re. 34,271, issued to Walder *et al.*; tetramer-free polymerized, pyridoxylated hemoglobin, as described in U.S. Patent Nos. 4,826,811 and 5,194,590, issued to Sehgal *et al.*; stable aldehyde polymerized hemoglobin, as described in U.S. Patent No. 4,857,636, issued to Hsia; hemoglobin covalently linked to sulfated glycosaminoglycans, as described in U.S. Patent No. 4,920,194, issued to Feller *et al.*; modified hemoglobin reacted with a high molecular weight polymer having reactive aldehyde constituents, as described in U.S. Patent No. 4,900,780, issued to Cerny; hemoglobin cross-linked in the presence of sodium tripolyphosphate, as described in U.S. Patent No. 5,128,452, issued to Hai *et al.*; stable, polyaldehyde polymerized hemoglobin, as described in U.S. Patent No. 5,189,146, issued to Hsia; and ß-cross-linked hemoglobin, as described in U.S. Patent No. 5,250,665, issued to Kluger *et al.*

Hemoglobin suspensions include hemoglobin in emulsions or emulsified hemoglobin solutions. Examples of hemoglobin suspensions include hemoglobin solutions which have a hemoglobin fraction encapsulated within water immiscible amphiphylic membranes, as described in U.S. Patent No. 4,543,130, issued to Djordjevich *et al.*; an emulsion of two aqueous phases to which stroma-free hemoglobin is added, as described in U.S. Patent No. 4,874,742, issued to Ecanow *et al.*; and a water-in-oil-in-water multiple emulsion of hemoglobin solution in a physiologically compatible oil, as described in U.S.

Patent Nos. 5,061,688 and 5,217,648, issued to Beissinger *et al.*

In a preferred embodiment, hemoglobin used in the method of invention is in the form of a polymerized hemoglobin blood-substitute described herein.

The composition of hemoglobin solutions, or blood-substitutes, preferred for use in the method of invention are sterile solutions having less than 0.5 endotoxin units/mL, a methemoglobin content that will not result in a significant reduction in oxygen transport/transfer capacity, a total hemoglobin concentration between about 2 to about 20 g Hb/dL, and a physiologic pH. In an even more preferred embodiment, the Hb solution has a total hemoglobin concentration between about 12 to about 14 g Hb/dL. Examples of preferred Hb solutions and blood-substitutes are described in the Examples below.

Typically, a suitable dose, or combination of doses, of hemoglobin is an amount of hemoglobin which, when contained within the blood plasma, will result in an increase in total hemoglobin concentration in a vertebrate's blood between about 0.1 to about 10 grams Hb/dL. A preferred dose for humans will increase total hemoglobin between about 0.5 to about 2 g Hb/dL. A preferred dose for dogs will increase total hemoglobin between about 3.5 to about 4.5 g Hb/kg body weight.

Hemoglobin can be administered into the circulatory system by injecting the hemoglobin directly and/or indirectly into the circulatory system of the vertebrate, by one or more injection methods. Examples of a direct injections methods include intravascular injections, such as intravenous and intra-arterial injections, and intracardiac injections. Examples of indirect injections methods include intraperitoneal injections, subcutaneous injections, such that the hemoglobin will be transported by the lymph system into the circulatory system,

injections into the bone marrow by means of a trocar or catheter. Preferably, the hemoglobin is administered intravenously.

The vertebrate being treated can be hypovolemic, normovolemic or hypervolemic prior to, during, and/or after infusion of the Hb solution. The hemoglobin can be directed into the circulatory system by methods such as top loading and by exchange methods.

Hemoglobin can be administered therapeutically, to treat hypoxic tissue within a vertebrate resulting from a reduced RBC flow in a portion of, or throughout, the circulatory system. Further, hemoglobin can be administered prophylactically to prevent oxygen-depletion of tissue within a vertebrate, which could result from a possible or expected reduction in RBC flow to a tissue or throughout the circulatory system of the vertebrate. Further discussion of the administration of hemoglobin to treat a partial arterial obstruction, therapeutically or prophylactically, or a partial blockage in microcirculation, is provided in Examples 10 and 11, respectively.

The invention will be further illustrated by the following examples.

## Example 1

## Synthesis of Stable Polymerized Hb Blood-Substitute

As described in U.S. Patent No. 5,296,465, samples of bovine whole blood were collected, mixed with a sodium citrate anticoagulant to form a blood solution, and then analyzed for endotoxin levels.

Each blood solution sample was maintained after collection at a temperature of about 2°C and then strained to remove large aggregates and particles with a 600 mesh screen.

Prior to pooling, the penicillin level in each blood solution sample was assayed with an assay kit purchased from Difco, Detroit, Michigan using the method entitled "Rapid Detection of Penicillin in Milk" to ensure that penicillin levels in the blood solutions were $\leq$ 0.008 units/ml.

The blood solution samples were then pooled and mixed with depyrogenated aqueous sodium citrate solution to form a 0.2% by weight solution of sodium citrate in bovine whole blood (hereafter "0.2% sodium citrate blood solution").

The 0.2% sodium citrate blood solution was then passed, in-series, through 800 μm and 50 μm polypropylene filters to remove large blood solution debris of a diameter approximately 50 μm or more.

The RBCs were then washed to separate extracellular plasma proteins, such as BSA or IgG, from the RBCs. To wash the RBCs contained in the blood solution, the volume of blood solution in the diafiltration tank was initially diluted by the addition of an equal volume of a filtered isotonic solution to diafiltration tank. The isotonic solution was filtered with a Millipore (Cat # CDUF 050 G1) 10,000 Dalton ultrafiltration membrane. The isotonic solution was composed of 6.0 g/l sodium citrate dihydrate and 8.0 g/l sodium chloride in water-for-injection (WFI).

The diluted blood solution was then concentrated back to its original volume by diafiltration through a 0.2 μm hollow fiber (Microgon Krosflo II microfiltration cartridge) diafilter. Concurrently, filtered isotonic solution was added continuously, as makeup, at a rate equal to the rate of filtrate loss through the 0.2 μm diafilter. During diafiltration, components of the diluted blood solution which were significantly smaller in diameter than RBCs, or are fluids such as plasma, passed through the walls of the 0.2 μm diafilter with the

filtrate. RBCs, platelets and larger bodies of the diluted blood solution, such as white blood cells, were retained with continuously-added isotonic solution to form a dialyzed blood solution.

During RBC washing, the diluted blood solution was maintained at a temperature between approximately 10°C to 25°C with a fluid pressure at the inlet of the diafilter between about 25 psi and about 30 psi to improve process efficiency.

RBC washing was complete when the volume of filtrate drained from the diafilter equaled about 600% of the volume of blood solution prior to diluting with filtered isotonic solution.

The dialyzed blood solution was then continuously pumped at a rate of approximately 4 lpm to a Sharples Super Centrifuge, Model # AS-16, fitted with a #28 ringdam. The centrifuge was operating while concurrently being fed dialyzed blood solution, to separate the RBCs from the white blood cells and platelets. During operation, the centrifuge rotated at a rate sufficient to separate the RBCs into a heavy RBC phase, while also separating a substantial portion of the white blood cells (WBCs) and platelets into a light WBC phase, specifically about 15,000 rpm. A fraction of the RBC phase and of the WBC phase were separately and continuously discharged from the centrifuge during operation.

Following separation of the RBCs, the RBCs were lysed to form a hemoglobin-containing solution. A substantial portion of the RBCs were mechanically lysed while discharging the RBCs from the centrifuge. The cell membranes of the RBCs ruptured upon impacting the wall of RBC phase discharge line at an angle to the flow of RBC phase out of the centrifuge, thereby releasing hemoglobin (Hb) from the RBCs into the RBC phase.

The lysed RBC phase then flowed through the RBC phase discharge line into a static mixer (Kenics 1/2 inch with 6 elements, Chemineer, Inc.). Concurrent with the transfer of the RBC phase to the static mixer, an equal amount of WFI was also injected into the static mixer, wherein the WFI mixed with the RBC phase. The flow rates of the RBC phase and the WFI into the static mixer are each at about 0.25 lpm.

Mixing the RBC phase with WFI in the static mixer produced a lysed RBC colloid. The lysed RBC colloid was then transferred from the static mixer into a Sharples Super Centrifuge (Model # AS-16, Sharples Division of Alfa-Laval Separation, Inc.) which was suitable to separate the Hb from non-hemoglobin RBC components. The centrifuge was rotated at a rate sufficient to separate the lysed RBC colloid into a light Hb phase and a heavy phase. The light phase was composed of Hb and also contained non-hemoglobin components with a density approximately equal to or less than the density of Hb.

The Hb phase was continuously discharged from the centrifuge, through a 0.45 $\mu m$ Millipore Pellicon Cassette, Cat # HVLP 000 C5 microfilter, and into a holding tank in preparation for Hb purification. Cell stroma were then returned with the retentate from the microfilter to the holding tank. During microfiltration, the temperature within the holding tank was maintained at 10°C or less. To improve efficiency, when the fluid pressure at the microfilter inlet increased from an initial pressure of about 10 psi to about 25 psi, microfiltration was complete. The Hb microfiltrate was then transferred from the microfilter into the microfiltrate tank.

Subsequently, the Hb microfiltrate was pumped through a 100,000 Millipore Cat # CDUF 050 H1 ultrafilter. A substantial portion of the Hb and water,

contained in the Hb microfiltrate, permeated the 100,000 Dalton ultrafilter to form a Hb ultrafiltrate, while larger cell debris, such as proteins with a molecular weight above about 100,000 Dalton, were retained and recirculated back into the microfiltrate tank. Concurrently, WFI was continuously added to the microfiltrate tank as makeup for water lost in the ultrafiltrate. Generally, cell debris include all whole and fragmented cellular components with the exception of Hb, smaller cell proteins, electrolytes, coenzymes and organic metabolic intermediates. Ultrafiltration continued until the concentration of Hb in the microfiltrate tank was less than 8 grams/liter (g/l). While ultrafiltering the Hb, the internal temperature of the microfiltrate tank was maintained at about 10°C.

The Hb ultrafiltrate was transferred into an ultrafiltrate tank, wherein the Hb ultrafiltrate was then recirculated through a 30,000 Dalton Millipore Cat # CDUF 050 T1 ultrafilter to remove smaller cell components, such as electrolytes, coenzymes, metabolic intermediates and proteins less than about 30,000 Daltons in molecular weight, and water from the Hb ultrafiltrate, thereby forming a concentrated Hb solution containing about 100 g Hb/l.

The concentrated Hb solution was then directed from the ultrafiltrate tank onto the media contained in parallel chromatographic columns (2 feet long with an 8 inch inner diameter) to separate the Hb by high performance liquid chromatography. The chromatographic columns contained an anion exchange medium suitable to separate Hb from non-hemoglobin proteins. The anion exchange media was formed from silica gel. The silica gel was exposed to $\gamma$-glycidoxy propyltrimethoxysilane to form active epoxide groups and then exposed to

dimethylaminoethanol ($HOCH_2CH_2N(CH_3)_2$) to form a quaternary ammonium anion exchange medium. This method of treating silica gel is described in the *Journal of Chromatography*, 120:321-333 (1976).

Each column was pre-treated by flushing the chromatographic columns with a first buffer which facilitated Hb binding. Then 4.52 liters of the concentrated Hb solution were injected into each chromatographic column. After injecting the concentrated Hb solution, the chromatographic columns were then washed by successively directing three different buffers through the chromatographic columns to produce a Hb eluate, by producing a pH gradient within the columns. The temperature of each buffer during use was about 12.4°C. The buffers were prefiltered through 10,000 Dalton ultrafiltration membrane before injection onto the chromatographic columns.

The first buffer, 20 mM tris-hydroxymethyl aminomethane (Tris) (pH about 8.4 to about 9.4), transported the concentrated Hb solution into the media in the chromatographic columns to bind the Hb. The second buffer, a mixture of the first buffer and a third buffer, with the second buffer having a pH of about 8.3, then adjusted the pH within chromatographic columns to elute contaminating non-hemoglobin components from the chromatographic columns, while retaining the Hb. Equilibration with the second buffer continued for about 30 minutes at a flow rate of approximately 3.56 lpm per column. The elute from the second buffer was discarded to waste. The third buffer, 50 mM Tris (pH about 6.5 to about 7.5), then eluted the Hb from the chromatographic columns.

The Hb eluate was then directed through a 0.22 $\mu$ Sartobran Cat # 5232507 G1PH filter to a tank wherein the

Hb eluate was collected. The first 3-to-4% of the Hb eluate and the last 3-to-4% of the Hb eluate were directed to waste.

The Hb eluate was then concentrated to approximately 100g Hb/L. The Hb eluate was further used if the eluate contained less than 0.05 EU/ml of endotoxin and contained less than 3.3 nmoles/ml phospholipids. To sixty liters of concentrated ultrapure eluate, which had a concentration of 100 g Hb/l, was added 9 l of 1.0 M NaCl, 20 mM Tris (pH 8.9) buffer, thereby forming an Hb solution with an ionic strength of 160 mM, to reduce the oxygen affinity of the Hb in the Hb solution. The Hb solution was then concentrated at 10°C, by recirculating through the ultrafilter, specifically a 10,000 Dalton Millipore Helicon, Cat # CDUF050G1 filter, until the Hb concentration was 110 g/l.

The Hb solution was then deoxygenated, until the $pO_2$ of the Hb solution was reduced to the level where $HbO_2$ content was about 10%, by recirculating the Hb solution at 12 lpm, through a 0.05 μm Hoechst-Celanese Corporati n Cat # G-240/40) polypropylene microfilter phase transfer membrane, to form a deoxygenated Hb solution (hereinafter "deoxy-Hb"). Concurrently, a 60 lpm flow of nitrogen gas was directed through the counter side of the phase transfer membrane. During deoxygenation, the temperature of the Hb solution was maintained between about 19°C and about 31°C.

Also during deoxygenation, and subsequently throughout the process, the Hb was maintained in a low oxygen environment to minimize oxygen absorption by the Hb and to maintain an oxygenated Hb (oxyhemoglobin or $HbO_2$) content of less than about 10% in the deoxy-Hb.

The deoxy-Hb, 60 l, was then diafiltered through an ultrafilter with 180 l of a storage buffer, containing

0.2 wt % N-acetyl cysteine, 33 mM sodium phosphate buffer (pH 7.8) having a $pO_2$ of less than 50 torr, to f rm a oxidation-stabilized deoxy-Hb. Prior to mixing with the deoxy-Hb, the storage buffer was depyrogenated with a 10,000 Dalton Millipore Helicon, Cat # CDUF050G1 depyrogenating filter.

The storage buffer was continuously added at a rate approximately equivalent to the fluid loss across the ultrafilter. Diafiltration continued until the volume of fluid lost through diafiltration across the ultrafilter was about three times the initial volume of the deoxy-Hb. The material may be stored at this point.

Prior to transferring the oxidation-stabilized deoxy-Hb into a polymerization apparatus, oxygen-depleted WFI was added to the polymerization reactor to purge the polymerization apparatus of oxygen to prevent oxygenation of oxidation-stabilized deoxy-Hb. The amount of WFI added to the polymerization apparatus was that amount which would result in a Hb solution with a concentration of about 40 g Hb/l, when the oxidation-stabilized deoxy-Hb was added to the polymerization reactor. The WFI was then recirculated throughout the polymerization apparatus, to deoxygenate the WFI by flow through a 0.05 μm polypropylene microfilter phase transfer membrane (Hoechst-Celanese Corporation Cat # 5PCM-108, 80 sq. ft.) against a counterflow of pressurized nitrogen. The flow rates of WFI and nitrogen gas, through the phase transfer membrane, were about 18 to 20 lpm and 40 to 60 lpm, respectively.

After the $pO_2$ of the WFI in polymerization apparatus was reduced to less than about 2 torr $pO_2$, the polymerization reactor was blanketed with nitrogen by a flow of about 20 lpm of nitrogen into the head space of the polymerization reactor. The oxidation-stabilized

deoxy-Hb was then transferr d into the polymerization reactor.

The polymerization was conducted in a 12 mM phosphate buffer with a pH of 7.8, having a chloride concentration less than or equal to about 35 mmolar which was produced by mixing the Hb solution with WFI.

The oxidation-stabilized deoxy-Hb and N-acetyl cysteine were subsequently slowly mixed with the cross-linking agent glutaraldehyde, specifically 29.4 grams of glutaraldehyde for each kilogram of Hb over a five hour period, while heating at 42°C and recirculating the Hb solution through a Kenics 1-1/2 inch static mixer with 6 elements (Chemineer, Inc.), to form a polymerized Hb (poly(Hb)) solution.

Recirculating the oxidation-stabilized deoxy-Hb and the glutaraldehyde through the static mixer caused turbulent flow conditions with generally uniform mixing of the glutaraldehyde with the oxidation-stabilized deoxy-Hb, thereby reducing the potential for forming pockets of deoxy-Hb containing high concentrations of glutaraldehyde. Generally uniform mixing of glutaraldehyde and deoxy-Hb reduced the formation of high molecular weight poly(Hb) (having a molecular weight above 500,000 Daltons) and also permitted faster mixing of glutaraldehyde and deoxy-Hb during polymerization.

In addition, significant Hb intramolecular cross-linking resulted during Hb polymerization as an effect of the presence of N-acetyl cysteine upon the polymerization of Hb.

After polymerization, the temperature of the poly(Hb) solution in the polymerization reactor was reduced to a temperature between about 15°C to about 25°C.

The poly(Hb) solution was then concentrated by recirculating the poly(Hb) solution through the

ultrafilter until the concentration of the poly(Hb) was increased to about 85 g/l. A suitable ultrafilter is a 30,000 Dalton filter (e.g., Millipore Helicon, Cat # CDUF050LT).

Subsequently, the poly(Hb) solution was then mixed with 66.75 g sodium borohydride, and then again recirculated through the static mixer. Specifically, for every nine liters of poly(Hb) solution, one liter of 0.25 M sodium borohydride solution was added at a rate of 0.1 to 0.12 lpm.

Prior to adding the sodium borohydride to the poly(Hb) solution, the pH of the poly(Hb) solution was basified by adjusting pH to a pH of about 10 to preserve the sodium borohydride and to prevent hydrogen gas formation. The pH of the poly(Hb) solution was adjusted by diafiltering the poly(Hb) solution with approximately 215 l of depyrogenated, deoxygenated 12 mM sodium borate buffer, having a pH of about 10.4 to about 10.6. The poly(Hb) solution was diafiltered by recirculating the poly(Hb) solution from the polymerization reactor through the 30 kD ultrafilter. The sodium borate buffer was added to the poly(Hb) solution at a rate approximately equivalent to the rate of fluid loss across the ultrafilter from diafiltration. Diafiltration continued until the volume of fluid lost across the ultrafilter from diafiltration was about three times the initial volume of the poly(Hb) solution in the polymerization reactor.

Following pH adjustment, sodium borohydride solution was added to the polymerization reactor to reduce imine bonds in the poly(Hb) solution to ketimine bonds and to form stable poly(Hb) in solution. During the sodium borohydride addition, the poly(Hb) solution in the polymerization reactor was continuously recirculated through the static mixer and the 0.05 μm polypropylene

microfilter phase transfer membrane to remove dissolved oxygen and hydrogen. Flow through a static mixer also provided turbulent sodium borohydride flow conditions that rapidly and effectively mixed sodium borohydride with the poly(Hb) solution. The flow rates of poly(Hb) solution and nitrogen gas through the 0.05 $\mu m$ phase transfer membrane were between about 2.0 to 4.0 lpm and about 12 to 18 lpm, respectively. After completion of the sodium borohydride addition, reduction continued in the polymerization reactor while an agitator contained therein rotated at approximately 75 rotations per minute.

Approximately one hour after the sodium borohydride addition, the stable poly(Hb) solution was recirculated from the polymerization reactor through the 30,000 Dalton ultrafilter until the stable poly(Hb) solution concentration was 110 g/l. Following concentration, the pH and electrolytes of the stable poly(Hb) solution were restored to physiologic levels to form a stable polymerized Hb blood-substitute, by diafiltering the stable poly(Hb) solution, through the 30,000 Dalton ultrafilter, with a filtered, deoxygenated, low pH buffer containing 27 mM sodium lactate, 12 mM NAC, 115 mM NaCl, 4 mM KCl, and 1.36 mM $CaCl_2$ in WFI, (pH 5.0). Diafiltration continued until the volume of fluid lost through diafiltration across the ultrafilter was about 6 times the pre-diafiltration volume of the concentrated Hb product.

After the pH and electrolytes were restored to physiologic levels, the stable polymerized Hb blood-substitute was then diluted to a concentration of 5.0 g/dl by adding the filtered, deoxygenated low pH buffer to polymerization reactor. The diluted blood-substitute was then diafiltered by recirculating from the polymerization reactor through the static mixer and a

100,000 Dalton purification filter against a filtered deoxygenated buffer containing 27 mM sodium lactate, 12 mM NAC, 115 mM NaCl, 4 mM KCl, and 1.36 mM $CaCl_2$ in WFI, (pH 7.8). Diafiltration continued until the blood-substitute contained less than or equal to about 10% modified tetrameric and unmodified tetrameric species by GPC when run under dissociating conditions.

The purification filter was run under conditions of low transmembrane pressure with a restricted permeate line. Following removal of substantial amounts of modified tetrameric Hb and unmodified tetrameric Hb, recirculation of the blood-substitute continued through the 30,000 Dalton ultrafilter until the concentration of the blood-substitute was about 130 g/l.

The stable blood-substitute was then stored in a suitable container having a low oxygen environment and a low oxygen in-leakage.

## Example 2
## Hemoglobin Blood-Substitute Storage

The hemoglobin blood-substitute, as prepared in Example 1, packaged in a 600 mL Stericon package, was overwrapped in a foil laminate package (KAPAK 50303, referred below as "foil"), Cryvac BYV200 or Cryovac P640B package. The construction of the KAPAK 50303 and Cryovac BYV200 containers are discussed in detail above. Cryovac P640B is a laminate material comprising a 0.6 mm Saran-coated, biaxially-oriented Nylon layer, 0.1 mm adhesive and a 2.0 mm linear low density polyethylene sealant layer. The oxygen permeability of the material is about 8 to 15 cc/100 sq. in/24 hours/atm/72°F/0% humidity. The packaged blood substitutes were maintained at room temperature for about 418 days with periodic sampling of the concentration and/or levels of N-acetyl-L-cysteine

(NAC), bis-N-acetyl-L-($HbO_2$) and methemoglobin (metHb). The results are set forth in Table II below.

Table II

Stability Data on Clear Overwraps Compound (800)

| Day | Over-wrap | NAC (%) | NAC² (%) | THb (g/dl) | $HbO_2$ (%) | metHB (%) |
|---|---|---|---|---|---|---|
| 0 | Foil | 0.1515 | 0.008 | 10.7 | 4.1 | -0.2 |
| 0 | BYV200 | 0.1586 | 0.0139 | 10.7 | 8.8 | -0.3 |
| 0 | P640B | 0.1274 | 0.0829 | 11.7 | 5.7 | 1.1 |
| 43 | Foil | 0.1688 | 0.0155 | 11.3 | 3.3 | -0.3 |
| 43 | BYV200 | 0.1509 | 0.0365 | 11.1 | 2.7 | 0.3 |
| 43 | P640B | 0.0507 | 0.1927 | 11.2 | 5.6 | 6.2 |
| 117 | Foil | 0.1721 | 0.0136 | 11.7 | 2.6 | 0.0 |
| 117 | BYV200 | 0.1433 | 0.0238 | 11.9 | 3.0 | 0.1 |
| 117 | P640B | 0.0022 | 0.2355 | 12.5 | 12.7 | 30.7 |
| 180 | Foil | 0.1818 | 0.0108 | 12.1 | 2.9 | -0.1 |
| 180 | BYV200 | 0.1674 | 0.0327 | 12.5 | 2.5 | 0.2 |
| 180 | P640B | N.D. | 0.2259 | 12.8 | 18.2 | 49.6 |
| 418 | Foil | 0.15 | 0.05 | 11.6 | 4.5 | 1.2 |
| 418 | BYV200 | 0.17 | 0.04 | 11.8 | 3.7 | 0.5 |
| 418 | P640B | N.D. | 0.19 | 12.0 | -1.3 | 92.3 |

The above experiment was essentially repeated wherein a hemoglobin blood-substitute was overwrapped in a foil laminate package (KAPAK 50303). The packaged blood substitutes were maintained at room temperature for about 24 months with periodic sampling of the concentration and/or levels of N-acetyl-L-cysteine (NAC), bis-N-acetyl-L-cysteine ($NAC_2$), total Hb (THb), oxygenated hemoglobin ($HbO_2$) and methemoglobin (metHb). The results are set forth in Table III below.

Table III

Stability Data on Clear Overwraps (Compound 800)

| Month | NAC (%) | $NAC_2$ (%) | THb (g/dl) | $HbO_2$ (%) | metHB (%) |
|---|---|---|---|---|---|
| 0 | 0.16 | 0.04 | 13.1 | 4 | 3 |
| 3 | 0.13 | 0.04 | 13.4 | 3 | 2 |
| 6 | 0.14 | 0.03 | 13.3 | 3 | 2 |
| 9 | 0.15 | 0.03 | 13.2 | 4 | 2 |
| 12 | 0.13 | 0.06 | 13.3 | 5 | 2 |
| 18 | 0.14 | 0.05 | 13.2 | 3 | 2 |
| 24 | 0.14 | 0.02 | 13.3 | 3 | 2 |

Example 3

Polymerized Hemoglobin Analysis

The endotoxin concentration in the hemoglobin product is determined by the method "Kinetic/ Turbidimetric LAL 5000 Methodology" developed by Associates of Cape Cod, Woods Hole, Massachusetts, J. Levin *et al.*, *J. Lab. Clin. Med.*, 75:903-911 (1970). Various methods were used to test for any traces of stroma for example, a precipitation assay, Immunoblotting, and enzyme-linked immunosorbent assay (ELISA) for a specific cell membrane protein or glycolipid known by those skilled in the art.

Particulate counting was determined by the method "Particulate Matter in Injections: Large Volume Injections for Single Dose Infusions", *U.S Pharmacopeia*, 22:1596, 1990.

To determine glutaraldehyde concentration, a 400 $\mu$l representative sample of the hemoglobin product was derivitized with dinitrophenylhydrazine and then a 100 $\mu$l aliquot of the derivative solution was injected onto a YMC AQ-303 ODS column at 27°C, at a rate of 1

ml/min., along with a gradient. The gradient consisted of two mobile phases, 0.1% trifluoroacetic acid (TFA) in water and 0.08% TFA in acetonitrile. The gradient flow consisted of a constant 60% 0.08% TFA in acetonitrile for 6.0 minutes, a linear gradient to 85% 0.08% TFA in acetonitrile over 12 minutes, a linear gradient to 100% 0.08% TFA in acetonitrile over 4 minutes hold at 100% 0.08% TFA in acetonitrile for 2 minutes and re-equilibrate at 45% of 0.1% TFA in water. Ultraviolet detection was measured at @360 nm.

To determine NAC concentration, an aliquot of hemoglobin product was diluted 1:100 with degassed sodium phosphate in water and 50 µl was injected onto a YMC AQ-303 ODS column with a gradient. The gradient buffers consisted of a sodium phosphate in water solution and a mixture of 80% acetonitrile in water with 0.05% TFA. The gradient flow consisted of 100% sodium phosphate in water for 15 minutes, then a linear gradient to 100% mixture of 80% acetonitrile and 0.05% TFA over 5 minutes, with a hold for 5 minutes. The system was then re-equilibrated at 100% sodium phosphate for 20 minutes.

Phospholipid analysis was done by a method based on procedures contained in the following two papers: Kolarovic *et al.*, "A Comparison of Extraction Methods for the Isolation of Phospholipids from Biological Sources", *Anal. Biochem.*, <u>156</u>:244-250, 1986 and Duck-Chong, C. G., "A Rapid Sensitive Method for Determining Phospholipid Phosphorus Involving Digestion With Magnesium Nitrate", *Lipids*, <u>14</u>:492-497, 1979.

Osmolarity was determined by analysis on an Advanced Cryomatic Osmometer, Model #3C2, Advanced Instruments, Inc., Needham, Massachusetts.

Total hemoglobin, methemoglobin and oxyhemoglobin concentrations were determined on a Co-Oximeter Model #482, from Instrumentation Laboratory, Lexington, Massachusetts.

$Na^+$, $K^+$, $Cl^-$, $Ca^{++}$, $pO_2$ concentration was determined by a Novastat Profile 4, Nova Biomedical Corporation, Waltham, Massachusetts.

Oxygen binding constant, $P_{50}$ were determined by a Hemox-Analyzer, TCS Corporation, Southhampton, Pennsylvania.

Temperature and pH were determined by standard methods known by those skilled in the art.

Molecular weight (M.W.) was determined by conducting gel permeation chromatography (GPC) on the hemoglobin products under dissociating conditions. A representative sample of the hemoglobin product was analyzed for molecular weight distribution. The hemoglobin product was diluted to 4 mg/ml within a mobile phase of 50 mM Bis-Tris (pH 6.5), 750 mM $MgCl_2$, and 0.1 mM EDTA. This buffer serves to dissociate Hb tetramer into dimers, that have not been cross-linked to other Hb dimers through intramolecular or intermolecular crosslinks, from the poly(Hb). The diluted sample was injected onto a TosoHaas G3000SW column. Flow rate was 0.5 ml/min. and ultraviolet detection was recorded at 280 nm.

The results of the above tests on veterinary (OXYGLOBIN™) and human (HEMOPURE™2) Hb blood-substitutes, formed according to the method of invention, are summarized in Tables IV and V, respectively.

Table IV

| PARAMETER | RESULTS |
|---|---|
| pH (18-22°C) | physiologically acceptable pH |
| Endotoxin | < 0.5 EU/ml |
| Sterility Test | Meets Test |
| Phospholipids[a] | <3.3 nm/ml |
| Total Hemoglobin | 12.0 - 14.0 g/dl |
| Methemoglobin | <15% |
| Oxyhemoglobin | ≤10% |
| Sodium, $Na^+$ | 145-160 mM |
| Potassium, $K^+$ | 3.5-5.5 mM |
| Chloride, $Cl^-$ | 105-120 mM |
| Calcium, $Ca^{++}$ | 0.5-1.5 mM |
| Boron | ≤10 ppm |
| Osmolality | 290-310 mOsm |
| Glutaraldehyde | <3.5 μg/ml |
| N-acetyl-L-cysteine | ≤0.2% |
| M.W. >500,000 | ≤15% |
| Unstabilized Tetramer | ≤5% |
| Particulate Content ≥10μ | <12/ml |
| Particulate Content ≥25μ | <2/ml |

a - measured in Hb before polymerization

Table V

| PARAMETER | RESULTS |
|---|---|
| pH (18-22°C) | Physiologically acceptable pH |
| Endotoxin | < 0.5 EU/ml |
| Sterility Test | Meets Test |
| Phospholipids[a] | <3.3 nm/ml |
| Total Hemoglobin | 12.0 - 14.0 g/dl |
| Methemoglobin | <15% |
| Oxyhemoglobin | ≤10% |
| Sodium, $Na^+$ | 145-160 mM |
| Potassium, $K^+$ | 3.5-5.5 mM |
| Chloride, $Cl^-$ | 105-120 mM |
| Calcium, $Ca^{++}$ | 0.5-1.5 mM |
| Boron | ≤10 ppm |
| Osmolality | 290-310 mOsm |
| Glutaraldehyde | <3.5 μg/ml |
| N-acetyl-L-cysteine | ≤0.2% |
| M.W. >500,000 | ≤15% |
| M.W. ≤ 65,000 | ≤10% |
| M.W. ≤32,000 | ≤5% |
| Particulate Content ≥10μ | <12/ml |
| Particulate Content ≥25μ | <2/ml |

a - measured in Hb before polymerization

In addition, evaluation of the blood-substitute, produced according to this method, showed that the blood-substitute also satisfactorily met a general safety test. In the general safety test, 2 mice and 2 guinea pigs were injected with blood-substitute, after which the test animals were monitored for 7 days. None of the animals injected with blood-substitute experienced a weight gain, a weight loss or death, thereby demonstrating the general safety of the blood-substitute.

## Example 4

## Stability Analysis of Stable Polymerized Hemoglobin Blood-Substitute

The stability of polymerized hemoglobin blood-substitute, produced according to the method of this invention, was evaluated over a period of 24 months at various storage temperatures. The specific storage temperatures evaluated were 2-8°C, room temperature (about 25°C) and 37°C. For storage at 2-8°C and at room temperature (RT), the stability results (provided in Table VI) show that the blood-substitute was stable for two years with only de minimis changes in the composition of the blood-substitute. Further, for storage at 37°C, the blood-substitute was stable for over one year. Blood-substitute stored at 37°C for 18 months, however, showed < 5 particles/ml at 25 $\mu$ and more than 11% Hb with a molecular weight greater than 500,000 Daltons.

In these analyses, total hemoglobin, methemoglobin and oxyhemoglobin concentrations were determined on a Co-Oximeter Model #482, from Instrumentation Laboratory, Lexington, MA.

Table VI

| Test and Specification | Initial | 24-Month Storage at 2-8°C | 24-Month Storage at RT | 12-Month Storage at 37°C |
|---|---|---|---|---|
| Package Integrity | Meets Test | Meets Test | Meets Test | Meets Test |
| Total Hb 13±1 g/dl | 12.6 | 12.8 | 12.8 | 12.8 |
| MetHb 10% | 3.1 | 1.8 | 2.0 | 1.4 |
| Glutaraldehyde <3.5μg/ml | N.D. | 0.03 | 0.03 | 0.01 |
| NAC ≤0.2% | 0.14 | 0.18 | 0.15 | 0.13 |
| Particulate Content <50/ml at ≥10 μ | Pass | Pass | Pass | Pass |
| <5/ml at ≥ 25 μ | Pass | Pass | Pass | Pass |
| Deep Purple Color | Pass | Pass | Pass | Pass |
| pH 7.6-7.9 | 7.8 | 7.8 | 7.8 | 7.8 |
| $HbO_2$ ≤10% | 4.5 | 2.3 | 2.3 | 3.1 |
| Osmolarity 290-310 mOsm | 303 | 298 | 300 | 299 |
| Molecular Weight >500kD ≤10% | 7.1 | 9.16 | 9.84 | 9.30 |
| ≤32kD ≤5% | 2.9 | 2.86 | 3.00 | 3.26 |

N.D. means not determined.

In addition, molecular weight was determined by conducting high performance size exclusion chromatography (HPSEC) on the blood-substitute. A representative sample of the blood-substitute was analyzed for molecular weight distribution. The hemoglobin product was diluted to 4 mg/ml within a mobile phase of 50 mM Bis-Tris (pH 6.5), 750 mM $MgCl_2$, and 0.1 mM EDTA. The diluted sample was injected onto a HPLC TosoHaas G3000SW column. Flow rate was 0.5 mL/minute and ultraviolet detection was set at @280 nm.

Package integrity was evaluated by performing a visual inspection of the packages, containing the blood-substitute, for leaks.

Example 5

Determination Of *In Vivo* Oncotic Effects in Canines

The purpose of this study was to determine the *in vivo* oncotic effects, specifically the volume of water drawn into the intravascular space per gram of hemoglobin administered, of veterinary (OXYGLOBIN™) Hb blood-substitute in splenectomized beagle dogs by measuring the expansion of plasma volume following a toploading dose. In addition, a comparable dose of (RHEOMACRODEX®-Saline), manufactured by Pharmacia, which is 10% Dextran 40 and 0.9% saline, was also determined.

Two dogs were entered into this study after a routine health screening and an acclimatization period of at least four weeks. The dogs were splenectomized at least 3 days before treatment. They were pre-anesthetized, with a combination of atropine and meperidine HCl, and anesthetized via inhalation of isoflurane. Lactated Ringer's solution was infused at 10-20 ml/kg/hr during the surgical procedure.

The dogs received the Hb blood-substitute (40 ml/kg) at 20 ml/kg/hr via a disposable cephalic catheter. Hematocrit was measured pre-dosing and at 1/4, 1/2, 1, 2, 3, 4 hours post-dosing or longer until the nadir of the hematocrit was established.

The dogs were splenectomized to ensure a constant plasma volume and RBC mass to allow accurate measurement of the change in plasma volume following dosing.

Calculation of the change in plasma volume was made using the following equation:

$$\Delta\%PV=\left\{\frac{Hct_1(1-Hct_2)}{Hct_2(1-Hct_1)}-1\right\}100$$

where PV is the plasma volume, $Hct_1$ is the initial hematocrit, and $Hct_2$ is the final hematocrit. This calculation was based on the change in hematocrit, assuming that the number of RBC's within the circulating blood volume and mean corpuscular volume remained constant.

As shown in Table VII, the nadir of the hematocrit occurred two hours post-dosing in both dogs. The mean corpuscular volume (MCV) remained stable throughout the study.

Table VII

| | Hematocrit (%) | | MCV (fL) | |
|---|---|---|---|---|
| Time (Hour) | Dog 3503C | Dog 14 Male | Dog 3503C | Dog 14 Male |
| 0 | 46 | 55 | 67.6 | 67.2 |
| 1/4 | 41 | 50 | 68.1 | 67.7 |
| 1/2 | 37 | 48 | 67.5 | 67.2 |
| 1 | 35 | 41 | 68.6 | 67.9 |
| 2 | 31 | 37 | 68.1 | 67.1 |
| 3 | 33 | 39 | 66.8 | 66.1 |
| 4 | 32 | 40 | 66.3 | 65.4 |

The volume of fluid drawn intravascularly post dosing was 6 ml/g hemoglobin and 9 ml/g hemoglobin for dogs 3503C and 14 male, respectively. The dose of synthetic colloid solution (Rheomacrodex®-Saline) was calculated based on a dose that causes a similar oncotic effect. Rheomacrodex draws approximately 22 ml fluid from the interstitium per gram administered intravenously.

The calculated comparable dose of Rheomacrodex was 14 ml/kg and 7 ml/kg for 30 ml/kg and 15 ml/kg Hb blood-substitute, respectively.

The volume of fluid drawn intravascularly by (Oxyglobin™) Hb blood-substitute was 8 ml $H_2O$/gram hemoglobin. Since the volume of the dose was 30 ml/kg, and the concentration of hemoglobin in the dose was 13 g/dl, the total amount of hemoglobin per dose was 3.9 g/kg and the total volume of fluid drawn into the intravascular space/dose by the Hb blood-substitute was 31.2 ml

The synthetic colloid solution draws in about 22 ml of water/gram of Dextran. The total amount of Dextran in the colloid solution per comparable dose of Hb blood-substitute is 1.4 g. Thus, the total volume of fluid

drawn into intravascular space/comparable dose of colloid solution is 14 ml.

## Example 6
## Canine Dose Response Study

This study was conducted to determine the drug effect and dose response of veterinary (OXYGLOBIN™) Hb blood-substitute of this invention, as compared to a synthetic colloid solution, of (RHEOMACRODEX®-Saline, Pharmacia) which is 10% Dextran 40 and 0.9% saline, with respect to arterial oxygen content relative to canine red blood cell hemoglobin and oxygen delivery in splenectomized beagle dogs 60 minutes and 24 hours following acute normovolemic hemodilution.

Acute normovolemic hemodilution is an experimental model that mimics a clinical condition of anemia due to surgical blood loss. Severe anemia (Hct = 9%, Hb = 3 g/dl) was produced by this method to cause an absolute requirement of oxygen carrying support. Oxygen delivery and oxygen content decrease precipitously with the massive bleeding.

In developing the normovolemic hemodilution model, it was found that treatment to restore oxygen delivery either by volume expansion alone, as was done for the control dogs, or by volume expansion in conjunction with an increase in the arterial oxygen content, as occurred for the dogs treated with hemoglobin solution, had to occur within approximately 10 minutes of reaching a hematocrit of 9% to avoid irreversible decreases in blood pressure and cardiac output which then resulted in death.

Two of 12 control dogs in this study died during or following dosing even though their vascular volume was expanded with Dextran 40 solution within 5 minutes of

reaching the targeted hematocrit. The death of these dogs is a reflection of the severity of the experimental model which in turn portrays the clinical condition of severe acute blood loss.

Thirty dogs were entered into this study after a routine health screening and acclimatization period of at least four weeks. Treatment was staggered using three replicates of dogs (A, B and C), each replicate containing one dog/sex/group. Dogs were randomly assigned to the 5 groups (6 dogs/group of 3 males and 3 females) 32 days before the first day of treatment. Dogs were assigned to their respective groups by block randomization based on body weight using a method which ensured equal distribution among groups. Males and females were randomized separately. Any dog with unacceptable pretreatment data, such as abnormal clinical signs or clinical pathology data, was replaced by a spare dog maintained under the same environmental conditions.

The test/control articles were administered by a single intravenous infusion. The rate of infusion was controlled by an infusion pump. The actual volume infused per hour depended upon the most recent body weight of each of the dogs.

The highest dose of hemoglobin solution was based upon the safe upper limit of acute cardiovascular effects due to volume expansion in normovolemic dogs. The mid-range dose was chosen to define the shape of the dose response curve. The lowest dose was based on the lower limit of clinically relevant dosing as defined by volume and hemodynamic effects in the dog.

Each dog was splenectomized at least 7 days before treatment to avoid effects on the experimental model of an increased circulatory RBC mass due to splenic contraction. On the day of treatment with hemoglobin solution, each dog was anesthetized by inhalation of

isoflurane and mechanically ventilated using room air with a tidal volume of 20-25 ml/Kg. The rate of ventilation was adjusted during the procedure to maintain arterial $pCO_2$ at approximately 40 mmHg. The end-expired concentration of isoflurane was measured and controlled to provide a valid comparison of anesthetic plane from dog to dog. The dogs were instrumented for monitoring of hemodynamic function and oxygen transport parameters. Placement of a flow-directed catheter in the pulmonary artery was confirmed by analysis of pressures and pressure tracings. A dual-lumen catheter, with thermodilution cardiac output capability, was placed in the femoral artery to provide an arterial line for blood pressure monitoring and blood withdrawal. A catheter was placed in the cephalic vein, or other vein if required, for volume replacement and test/control article administration.

Each dog received an intramuscular injection of antibiotics once daily (Procaine penicillin G) prophylactically for one day prior to surgery, on the day of surgery and for 3 days following the splenectomy. V-Sporin, a topical antibiotic (Polymyxin B, Bacitracin, Neomycin) was applied to the surgical site once daily, as needed.

Following instrumentation, hemodynamic stabilization to reach a $pCO_2$ of approximately 40 mm Hg and collection of baseline measurements were performed. A model of acute normovolemic hemodilution was then produced by bleeding the dogs and simultaneously replacing approximately 1.6 to 2.3 times the volumes withdrawn with Lactated Ringer's Solution to maintain isovolemic status. Isovolemic status was achieved by maintaining pulmonary artery wedge pressure at approximately baseline values. The blood withdrawal/volume replacement took

approximately 45 to 90 minutes until the hemoglobin concentration was approximately 30 g/l (3.0 g/dl). Lactated Ringer's Solution was infused rapidly using a gravity intravenous set and a pressure cuff around the infusion bag. If the arterial systolic blood pressure was ≤ 50 mmHg for more than 5 minutes following the induction of acute anemia and prior to the start of dosing, the dog was rejected and replaced by a spare dog maintained under the same environmental conditions.

Doses of colloid control and hemoglobin solution were administered as stated in Table VIII. Hemodynamic measurements were performed pre-bleed, pre-dose, immediately following dosing, and at 60 minutes and 24 hours following dosing. After the 60 minute measurement, the dog recovered from anesthesia and was instrumented again for hemodynamic measurements, performed at 24 hours following dosing.

Table VIII

| Group | Test Article | Dose Volume ml/Kg | Dose Rate ml/Kg/h | Animals/Group Males | Females |
|---|---|---|---|---|---|
| 1 | Colloid control (mid dose) | 14 | 20 | 3 | 3 |
| 2 | Colloid control (low dose) | 7 | 20 | 3 | 3 |
| 3 | Hb blood-substitute (low dose) | 15 | 20 | 3 | 3 |
| 4 | Hb blood-substitute (mid dose) | 30 | 20 | 3 | 3 |
| 5 | Hb blood-substitute (high dose) | 45 | 20 | 3 | 3 |

All hemodynamic parameters were statistically analyzed by either analysis of variance (ANOVA) or analysis of covariance (ANCOVA) with either the pre-bleeding or pre-dosing value as the covariate. Specific linear contrasts were constructed to test for the effects

of volume of the solution administered, the effect of Hb blood-substitute (drug effect), and the dose response of the Hb blood-substitute (dose effect). These tests were performed only for parameters for which the difference among experimental groups was statistically significant at the 0.05 level. Comparisons of specified variables at selected time points were performed by paired t-tests in each group.

Arterial oxygen content was one criterion of efficacy in this study. Arterial oxygen content is a measure of the oxygen carrying capacity of cellular and plasma hemoglobin and dissolved oxygen in the plasma. In the absence of plasma hemoglobin, arterial oxygen content is calculated from the amount of oxygen carried by saturated cellular hemoglobin and the partial pressure of inspired oxygen. Because plasma hemoglobin was expected to contribute significantly to oxygen content in this study, oxygen content was measured directly using a LexO2Con-K instrument (Chestnut Hill, MA). Oxygen enriched air was not administered during the experiment because it was unnecessary and to avoid the confounding effects of an increased inspired oxygen concentration on the measurement of arterial oxygen content.

Mean arterial and venous oxygen contents decreased approximately four and eight times, respectively in all groups following induction of anemia. Arterial oxygen content increased significantly 60 minutes following dosing compared to pre-dosing values in all Hb blood-substitute treated groups and remained significantly increased at 24 hours following dosing in the mid and high dose groups. Arterial or venous oxygen content did not change following dosing in either control group.

As shown in Figure 2, arterial oxygen content was significantly increased in Hb blood-substitute treated groups compared to control groups at 60 minutes and 24

hours foll wing dosing. A linear dose response was seen at 60 minutes and 24 hours foll wing dosing. A significant volume effect was detected for arterial oxygen content 60 minutes following dosing.

Venous oxygen content also significantly increased in Hb blood-substitute treated groups compared to controls at 60 minutes and 24 hours following dosing. The increase showed a linear dose response at 60 minutes following dosing but not at 24 hours.

The dose effect observed for Hb blood-substitute treated groups in arterial-venous (A-V) oxygen content difference at 60 minutes following dosing was attributed to significant volume effects based on the absence of a drug effect and similar observations of volume effects in control groups at 60 minutes following dosing. Hb blood-substitute treated groups showed a significant increase in A-V oxygen difference at 24 hours compared to colloid controls, with a significant linear dose response. The A-V difference must be interpreted in view of the cardiac output. At 24 hours following dosing, the A-V difference in the control groups was significantly lower than that of the Hb blood-substitute treated groups. One possible explanation for this difference is that the control group dogs had to rely on a higher cardiac output to meet the oxygen consumption needs of peripheral tissues. The Hb blood-substitute treated groups maintained a large enough A-V difference at 24 hours following dosing to meet peripheral tissue needs without cause for an increased cardiac output.

In addition to arterial oxygen content, total arterial oxygen content normalized relative to the contribution of canine RBC hemoglobin ($CaO_2$/g RBC Hb) was examined in this study. This comparison was made to demonstrate differences in arterial oxygen content among

dosing groups since the RBC hemoglobin was constant in all gr ups. The potential correlation of plasma or total hemoglobin concentration and arterial oxygen content would provide a useful clinical measure of efficacy. As shown in Figure 3, at 60 minutes and 24 hours following dosing, all Hb blood-substitute treated groups (except the low dose group at 24 hours) showed a significant increase in $CaO_2$/g RBC hemoglobin to pre-dosing values. Arterial oxygen content relative to that contributed by RBC hemoglobin did not differ significantly in the colloid controls between pre-dose and 60 minutes or 24 hours following dosing.

Total arterial oxygen content relative to that contributed by red blood cell hemoglobin significantly increased in Hb blood-substitute treated groups compared to colloid controls at 60 minutes following dosing with a significant linear dose response. A significant dose effect also occurred at 24 hours following dosing with a significant linear dose response, but the drug effect was not quite significant ($P<0.06$).

Oxygen delivery was another criterion of efficacy. Oxygen delivery is calculated based on arterial oxygen content and cardiac output. Therefore, oxygen delivery is affected by all the physiologic factors which influence cardiac output. The control chosen for this study was the synthetic colloid (RHEOMACRODEX®-Saline, Pharmacia) which is 10% Dextran 40 and 0.9% saline, as it expands intravascular volume and is not known to carry oxygen. The control provided a comparison of equivalent volume expansion to the colloidal properties of the hemoglobin in Hb blood-substitute.

Because each dose of Hb blood-substitute was expected to demonstrate a distinct volume effect, two doses of dextran solution were used as controls for the

volume effect so the data would reflect only the drug effect of different doses. This comparison was made for the low and mid doses. The doses of colloid controls were selected based on those doses of Dextran 40 which provided an equivalent comparison of the *in vivo* oncotic effects of the low and mid-dose test articles, as determined from the results of Example 5.

The volume effect was defined statistically using the difference in means between the colloid mid dose (14 ml/kg) and the colloid low dose (7 ml/kg). The drug effect was determined by comparing each Hb blood-substitute treated group to its corresponding colloid control. A linear dose response was established when a statistically significant difference was seen between the low and high dose Hb blood-substitute treated groups.

Oxygen delivery was calculated according to the equation: $DO_2 = CO \times CaO_2 \times 10/kg$ where CO is the cardiac output and $CaO_2$ is the arterial oxygen content. As expected, following induction of anemia in all treatment groups, a two to three fold mean decrease in $DO_2$ occurred in all groups. The oxygen content decreased sufficiently that the maintenance of baseline oxygen consumption had to result from an increase in cardiac output and increased extraction of oxygen, resulting in a lower venous oxygen content. As shown in Figure 4, oxygen delivery increased approximately 30% in the low dose Hb blood-substitute treated group and greater than 100% in the mid and high Hb blood-substitute treated groups at 60 minutes following dosing compared to pre-dosing values. The difference was significant for all three dosing groups ($p<0.05$). The control groups showed no significant differences over this time. At 60 minutes following dosing, $DO_2$ differed significantly among all groups with significant drug and dose effects with a

linear dose response. At 24 hours, no difference in oxygen delivery was noted among groups. The improvement in oxygen delivery at 60 minutes following dosing for all Hb blood-substitute treated groups, as compared to their corresponding colloid controls, was due primarily to a dose related increase in arterial oxygen content in addition to a modest increase in cardiac output.

Oxygen consumption was calculated according to the equation: $VO_2 = CO \times CaO_2 \times 10$ kg. A two to three fold mean decrease in $DO_2$ occurred in all groups following the induction of anemia. No statistically significant differences were noted among Hb blood-substitute treat d or control groups or within a group when comparing pre-dosing to post-dosing values.

The Oxygen Extraction Ratio ($VO_2/DO_2$) for all groups showed an approximately three fold increase following induction of anemia. Oxygen extraction ratios were significantly decreased in a dose dependent manner in all Hb blood-substitute treated groups at 60 minutes following dosing compared to control groups. No significant differences were noted between Hb blood-substitute treated and control groups at 24 hours following dosing.

Mean cardiac output increased between 10% and 39% in all groups following induction of anemia. Cardiac output was significantly increased at 24 hours following dosing compared to pre bleeding values in the colloid control groups but not in the Hb blood-substitute treated groups. A significant volume effect which contributed to significant differences in cardiac output between colloid low and mid dose groups was evident at 60 minutes post-dosing. The increase in cardiac output was likely related to an increased stroke volume due to expansion of the intravascular volume following dosing or increased

sympathetic tone due to the stress of severe anemia. A significant dose response between Hb blood-substitute low and high dose groups was apparent at 60 minutes, but not at 24 hours following dosing. No difference in cardiac output between Hb blood-substitute treated and colloid control groups was seen at 60 minutes or 24 hours following dosing.

Pulmonary artery wedge pressure (PAWP) did not change significantly during the induction of anemia. PAWP decreased significantly in the low dose colloid group and remained unchanged in the mid dose colloid group 60 minutes following dosing compared to pre dosing values. The PAWP in the mid and high dose Hb blood-substitute treated groups increased significantly in a linear dose response compared to pre-dosing values at 60 minutes following dosing. The increased PAWP reflected a dose dependent increase in intravascular volume at 60 minutes following dosing. No significant drug effect was detected between Hb blood-substitute treated and control groups at 60 minutes or 24 hours following dosing. A significant volume effect was detected in the colloid control groups at 60 minutes following dosing.

Systolic, diastolic and mean arterial blood pressure decreased significantly in all groups following induction of anemia, then increased significantly immediately following dosing. The decrease in systolic arterial blood pressure after the induction of anemia was likely related to a decrease in peripheral vascular resistance due to decreased blood viscosity, a consequence of anemia. At 60 minutes following dosing, the systolic, diastolic, and mean arterial blood pressures of both colloid control groups did not differ significantly from pre-dosing values. The systolic, diastolic, and mean pressures of the low dose colloid control increased significantly compared to pre-dosing values at 24 hours

following dosing. In contrast, the increase in systolic, diastolic, and mean pressures was statistically significant in all Hb blood-substitute treated groups at 60 minutes and 24 hours following dosing compared to pre-dosing values. The systolic, diastolic and mean blood pressures of Hb blood-substitute treated groups were significantly higher than corresponding colloid control groups at 60 minutes following dosing, but not at 24 hours.

Significant increases in systolic, diastolic and mean pulmonary arterial pressures were observed in the mid and high dose Hb blood-substitute treated groups 60 minutes post dosing compared to pre dosing values. The increases persisted at 24 hours post-dosing in the mid Hb blood-substitute treated group for pulmonary diastolic arterial pressure. Additionally the low-dose colloid group showed a statistically significant increase at 24 hours post-dosing compared to pre-dosing values for mean pulmonary artery pressure. This increase was considered clinically significant. The increases in systemic arterial systolic and diastolic blood pressure 60 minutes following dosing of Hb blood-substitute, compared to pre-dosing values, were a direct drug effect of the Hb blood-substitute. The diastolic pressure remained unchanged in the colloid control groups which was probably a result of a decreased peripheral vascular resistance.

No significant differences were found between Hb blood-substitute treated and control groups for pulmonary systolic arterial pressure at either 60 minutes or 24 hours post-dose. In contrast, pulmonary diastolic and mean arterial pressures were significantly different with regard to volume, drug, and dose effects at 60 minutes post dosing, but not at 24 hours.

Total hemoglobin decreased approximately four times or greater with bleeding. Hb blood-substitute treated

groups showed a dose dependent increase in total hemoglobin compared to corresponding colloid control groups at 60 minutes and 24 hours following dosing.

Plasma hemoglobin concentrations significantly increased in a dose dependent manner in Hb blood-substitute treated groups compared to corresponding colloid control groups at 60 minutes and 24 hours following dosing. The increases in plasma and total hemoglobin concentrations following dosing in all Hb blood-substitute treated groups, as compared to their corresponding colloid controls, were attributable to the hemoglobin content of Hb blood-substitute. The dose dependent significant increase persisted for 24 hours, correlating with the persistent increase in arterial oxygen content.

In summary, the response to treatment with the Hb blood-substitute was linear, i.e., at 60 minutes following dosing, the higher the dose of Hb blood-substitute the greater the improvement in oxygen delivery and hemodynamics compared to corresponding colloid controls. Sustained arterial oxygen content and normal clinical signs, while breathing room air, support a beneficial biological effect of Hb blood-substitute lasting 24 hours in the 30 ml/kg and 45 ml/kg dose Hb blood-substitute treated groups. The clearance of Hb blood-substitute likely accounts for the changes seen in oxygen delivery and hemodynamic effects at 24 hours following dosing. In conclusion, results from this study support selection of a dose ranging from 30 to 45 ml/kg. Both of these dosing groups showed statistically significant differences from corresponding colloid control groups in the parameters of efficacy and the dose response was linear.

The clinical rationale of this dosing range is based on the fact that a severely anemic dog (e.g., hematocrit

<15% with marked clinical signs) would benefit from a higher dose as demonstrated by the linear dose response of improved arterial oxygen content and oxygen delivery. However, a more conservative dose would be indicated for a dog which may be predisposed to intravascular volume overload. The dose dependent transient increase in pulmonary artery wedge pressure and pulmonary arterial pressures seen 60 minutes following dosing in Hb blood-substitute treated groups would limit the use of a higher dose in this population of dogs. Therefore a dosing range of 30-45 ml/kg would be effective in a broad population of dogs in which the degree of anemia and intravascular volume status are defined.

Example 7

Human Dose Response Study

This study was conducted to evaluate the safety and tolerance of increasing rates of intravenous administration of Hb blood-substitute (hereinafter HBOL) upon hemodynamic, neuroendocrine and hematologic parameters in humans. The test subjects were normal healthy adult males (70-90 kg) between the ages of 18-45 years. During the study, the test subjects were on controlled isocaloric diets of 55% carbohydrates, 30% fat (polyunsaturated to saturated fat ratio of 2:1), 15% protein and 150 mEq of sodium per day. Fluid intake was at least 3000 mls/day with caffeine containing beverages avoided. Also concomitant use of medication was avoided. Further no alcohol or tobacco were used by the test subjects during the study.

The 12 subjects studied, were divided into three test groups. In each test group, three subjects received HBOL and one served as a control, receiving Ringer's lactat . Each test group had different rates of HBOL

infusion. The study was conducted as a single-blind, rate escalati n study over a thirty day interval.

On Day 1 of the study, during the inpatient phase, each subject had a small gauge arterial catheter inserted in the radial artery of the non-dominant hand. The insertion location was cleansed with an antiseptic solution (alcohol and/or iodine) and then a small amount of 1%-2% Lidocaine anesthetic solution was subcutaneously injected over the site of the radial artery. The arterial catheter was inserted to monitor blood pressure and to facilitate blood gases evaluations. One to two hours later, all subjects had one large-bore intravenous catheter (16-gauge needle in antecubital fossa) placed in a vein in one arm. Each subject then had a phlebotomy of 750 ml (1.5 units) of whole blood drawn in less than 15 minutes, which was then followed with isovolemic hemodilution by the infusion of 2250 ml of Ringer's lactate over a 2 hour period.

Forty-five grams (346 ml) of HBOL were then intravenously infused using sterile technique, in series through a standard 80 micrometer blood filter, a 5 micrometer filter, and the large-bore intravenous catheter in the arm vein, into each subject in the test groups 1, 2 and 3 at the rates of 0.5 gm/minute, 0.75 gm/minute and 1.0 gm/minute, respectively.

Simultaneously, each subject had invasive monitoring by radial artery catheter, serial pulmonary function tests, cardiac function evaluation and multiple hematology, chemistry and urinalysis laboratory tests which were routinely and frequently performed over the first 28 hours after commencing HBOL infusion.

Subsequently, in the outpatient phase (Days 2-29), laboratory studies, vital signs, ECGs and medical events were taken daily for the first four days post-discharge and then on a weekly basis for a month.

Hemodynamics were remarkable for generally higher values f r systolic, diastolic and mean arterial pressure in the HBOL-treated groups (after infusion) than controls during Day 1. Although there was marked variability in the blood pressure data commensurate with patient activity (e.g., during meals or when using the bathroom) and diurnal rhythm, HBOL-treated subjects generally had values for the systolic blood pressure (about 5-15 mm Hg), diastolic blood pressure (about 5-10 mm Hg) and mean arterial pressure (about 10 mm Hg) greater than controls only during the course of Day 1. Values tended to reach peak effects between Hours 8-12 with return to baseline during sleep and upon removal of the arterial catheter. Pulse was generally about 10 beats lower in all HBOL-treated groups compared to controls during Day 1. The nadir of pulse decline was seen within the first 15 minutes of the infusion. Values were similar in all test groups after hour 24.

Cardiac index declined about 1-2 $l/min/m^2$ during the first hour of infusion remained up to 1 $l/min/m^2$ lower than controls through hour 4, and then it returned to baseline by hour 4. Cardiac index also increased during times of patient activity (as above).

Total peripheral resistance paralleled blood pressure changes, however, values returned to baseline within two hours. The transient increase in systemic blood pressure with an increase in total peripheral resistance and decrease in cardiac index is not unexpected. It is important to note that there was no difference in the rate of administration and the magnitude of these hemodynamic responses and that no intervention was indicated.

The pulmonary function tests (including multiple determinations of spirometry and lung volumes) and

arterial blood gas measurements were unremarkable. What was noteworthy was the enhanced diffusion capacity that was seen in the HBOL-treated groups. The 10-15% increase in diffusion capacity was statistically significant compared to a 10% decrease in the controls for up to 24 hours. These findings are particularly important because of the magnitude of phlebotomy and hemodilution that all of the groups underwent.

In hematological studies, other than the expected, transient decline in hemoglobin, hematocrit, red cell count and serum proteins with the phlebotomy and hemodilution procedures, the hematology and serum chemistry laboratory tests were unremarkable. Exceptions were serum iron and ferritin which showed peak values by Hours 6 and 48, respectively, after HBOL was given.

The serum chemistry measurements were unremarkable, with the exception of one subject (#10) who had transient increases in serum transaminases and lipase. It is important to note that this subject did not have any clinically significant concomitant medical events (e.g., dysphagia or abdominal pain) commensurate with the time of the elevation of these enzymes. The exact etiology of these laboratory abnormalities is unclear, but previous studies suggest that transient subclinical spasm of the sphincter of Oddi or other portions of the hepatobiliary and pancreatic ductal systems may be involved. It is important to note that these changes were transient (and unaccompanied by abdominal discomfort) and without apparent sequelae. No significant change was noted in Subject #10's post-dose ultrasound of the gall bladder.

Urinalysis was unremarkable throughout the study. There was no detectable urinary hemoglobin in the subjects during the study. In addition, creatinine clearance was slightly higher, as expected, during the hemodilution period), urinary adenosine deaminase binding

protein, electrolytes (sodium, potassium, chloride), iron, microalbumin, NAG (N-acetyl-beta-glucosaminidase) and urinary urea nitrogen were unremarkable.

No apparent changes in the majority of the pharmacokinetic parameters were observed as a function of administration rate. Sequential blood specimens and cumulative urine specimens were collected prior to and following initiation of infusion of HBOL for size exclusion (gel filtration) chromatographic (SEC) analysis of total hemoglobin and apparent molecular weight fractions of hemoglobin. Only sporadic plasma dimmer fraction concentrations were observed precluding any pharmacokinetic analysis. The only statistically significant differences ($p < 0.05$) were observed in the tetramer volume of distribution (decreases with increases in rate), tetramer maximum concentration achieved (increases with increases in rate) and the time of the tetramer maximum concentration occurrence (decreases with increases in rate).

The observed medical events were consistent with expected findings related to phlebotomy (e.g., vasovagal episode), multiple pulmonary function tests (aerophagia, eructation or abdominal "gas"), arterial line insertion (e.g., pain or tingling over the site), or abdominal discomfort (e.g., associated with the ingestion of the iron supplement). Although there seemed to be a background of nonspecific, transient abdominal "gas" there were no cases of overt abdominal pain or dysphagia. In addition there was no correlation of these symptoms with any alterations in serum transaminases or lipase.

In summary, HBOL was well tolerated. Although there were small transient increases in blood pressure and total peripheral resistance with commensurate decline in cardiac index during the first two hours of the infusion, the hemodynamics were unremarkable. The increase in

diffusion capacity was significantly higher in the HBOL-treated gr ups than controls during the first 24 hours.

## Example 8

### Effects of HBOL on Humans in Graded Bicycle Exercise Testing

This study was conducted to evaluate the exercise capacity of subjects given autologous transfusion of HBOL. Specific endpoints included pulmonary function (e.g., diffusion capacity and lactic acid levels and $pO_2$), hemodynamics (e.g,, heart rate, cardiac index and blood pressure) and exercise tolerance (e.g., duration, workload and anaerobic threshold). The subjects were six normal healthy male humans, ages 18-45 years. One subject was replaced in the study due to failure to obtain the volume of phlebotomy in less than 15 minutes. The study was conducted as a randomized, single-blind, two-way crossover study.

All subjects had phlebotomy of 750 ml followed by Ringer's Lactate [3:1] and either an autologous transfusion (ATX) or 45 gms of HBOL. The ATX or HBOL was given at 0.5 gm/min for 90 minutes. Bicycle exercise stress tests were done on the day prior to phlebotomy and approximately 45 minutes after the infusion of ATX or HBOL. The same procedures were repeated one week later and subjects were crossed over to the opposite treatment.

On the day of dosing (Days 1 and 8), all subjects had insertion of an arterial line in one radial artery, attachment to cardia telemetry and impedance cardiography and then phlebotomy (PBX) of 750 ml of whole blood (< 15 minutes). This was followed by an infusion of 2250 ml of Ringer's lactate (RL) over two hours (the isovolemic hemodilution phase). Subjects then received either HBOL (45 gms [about 346 - 360 ml] at a rate of 0.5 gm/min over

90 minutes) or an ATX (110 -120 gms f hemoglobin [about 750 ml] at the same rate and duration a the HBOL). The BEST was done about 45 minutes after the end of either infusion. Serial measurements of arterial blood gases, hematology, chemistry and urine tests were made intensively during the 24 hour period on Days 1 and 8. Serial follow-up was done on an outpatient basis between the dosing and for one month after all dosing was complete.

Subjects were able to exercise to similar levels during HBOL and ATX periods. The oxygen uptake ($VO_2$) and carbon dioxide production ($VCO_2$) at anaerobic threshold were nearly identical. The actual workload in METS, watts, pulse (as a % of maximum pulse), time to anaerobic threshold, tidal volume (VT) and minute ventilation (VE) were also similar. Arterial blood gas values were similar during the HBOL and ATX periods. The small reductions in pH and bicarbonate with increase in lactic acid is consistent with expected findings at anaerobic threshold. The results of these bicycle tests showed that exercise capacity (defined as time and workload to reach anaerobic threshold) was similar at baseline and after infusions of either autologous transfusion or HBOL. Specifically, hemodynamics were remarkable for slightly higher values (approximately 5 mmHg) during the HBOL period for systolic, diastolic and mean arterial pressure. Commensurate with the increase in blood pressure was an increase in total peripheral resistance, generally within the first 4 hours. Cardiac index declined during the HBOL period (- 0.5 l/min/$M^2$). Pulse was about 5-10 beats lower during the HBOL than the ATX period. These findings have been observed in the HBOL studies and have been of little clinical concern.

Pulmonary function tests were unremarkable except for a 14% increase above baseline in diffusion capacity after the ATX and HBOL infusions. Subjects were able to achieve similar exercise capacity during HBOL and ATX periods. Arterial blood gas measurements during peak exercise (anaerobic threshold) were similar in both periods, but arterial $pO_2$ tended to be higher during the HBOL period. Plasma lactic acid levels were lower during the HBOL than ATX period. Resting metabolic art measurements indicated that oxygen consumption, carbon dioxide production and metabolic energy expenditure were greater during the HBOL than ATX period. The comparison as mentioned above is roughly one gram of HBOL to 3 grams of ATX. The diffusion capacity coupled with the observations about $VO_2$ and $VCO_2$ indicate that more oxygen is being delivered to the tissue level per gram of HBOL than ATX. It is commonly held that the diffusion capacity varies directly with the hemoglobin level, however, there is a suggestion that 1 gram of plasma hemoglobin may increase diffusion capacity as much as 3 gms of RBC hemoglobin.

Laboratory studies were notable for small, but transient increases in ALT, AST, 5'-nucleotidase, lipase and creatine kinase during the HBOL period. There were no abnormal urinary finding.

Hematological studies were consistent with those in Example 7.

The observed medical events were consistent with expected findings related to the phlebotomy (e.g., vasovagal episode), multiple pulmonary function tests (eructation or abdominal "gas"), arterial line insertion (e.g., pain or tingling over the insertion site) or numerous everyday complaints that one might observe in normal subjects over the course of a month (e.g.,

headache, upper respiratory tract infection or cold). The one subject (Subject #105) that had abdominal "gas" and pressure in the mid-epigastrium, but without dysphagia is suggestive of other gastrointestinal complaints that have been observed in previous HBOL studies. L-arginine was used as a therapeutic measure based on the concept that hemoglobin can interfere with endogenous nitric oxide function (nitric oxide is integral in the relaxation of gastrointestinal smooth muscle, especially in the esophagus and intestines). L-arginine is the substrate upon which nitric oxide synthase produces nitric oxide. Theoretically, if one has a reduction in nitric oxide from the hemoglobin (perhaps binding of heme to nitric oxide), then administration of L-arginine might be of benefit. Apparently the subject did get marked by transient relief from his symptoms with the L-arginine for about two hours. This is not an unexpected finding because the plasma half-life of L-arginine is about an hour. Unfortunately, some of the side effects (nausea and vomiting) occurred and the infusion was stopped. We elected to give him a two doses of an anticholinergic, antispasmodic drug, hyoscyamine. This apparently continued to reduce the symptoms of abdominal "gas" and pressure. The subject had no further complaints or sequelae.

In summary, HBOL was associated with improved oxygen delivery and utilization during exercise and at rest. HBOL produced a similar spectrum of hemodynamic, safety laboratory results, pharmacokinetics and medical events to what has previously been observed. Intervention with L-arginine may produce a reversal of gastrointestinal symptoms, but its use was limited by nausea and vomiting. However, the use of anticholinergic therapy might be of

value in the treatment for the gastrointestinal symptoms that are encountered.

## Example 9

## Study of Tissue Oxygenation from Infusing Polymerized Hemoglobin Solution after Hemodilution

In this study, regional tissue oxygenation levels were measured in the left hind limb muscle (m. gastrocnemius), within an 8-dog experimental group, to determine the effects of infusion of polymerized hemoglobin solution upon animals made anemic by isovolemic hemodilution with a non-oxygen bearing solution.

Regional tissue oxygen partial tensions were determined, using a Sigma-$pO_2$-Histograph (Model No. KIMOC 6650, Eppendorf-Netherler-Hinz GmbH, Hamburg, Germany), to measure at least 200 local $pO_2$ values in the skeletal musculature distal to the exposed femoral artery, and then display the $pO_2$ values in a histogram for each measurement point.

At each measurement point, the Eppendorf $pO_2$-Histograph measured oxygen partial pressure polarographically with an oxygen needle probe having a spring steel casing containing a glass-insulated, teflon-coated gold microcathode. The oxygen needle probe was polarized with -700 mV towards an Ag/AgCl anode, which was attached to the skin near the site of the oxygen needle probe insertion. The resulting current was proportional to the oxygen partial pressure at the electrode tip, thus giving a measurement of local tissue oxygenation.

Regional oxygenation measurements were obtained automatically with the aid of a microprocessor-controlled manipulator, which moved the oxygen needle probe through the tissue in a series of "pilgrim steps", each typically

consisting of a forward motion of 1 mm followed by a backward motion of 0.3 mm, to relieve compression of the tissue from the forward motion, with subsequent $pO_2$ value sampling. At the end of each tissue oxygenation measurement, the needle probe was moved to a new tissue location, such that each measurement was performed only in undisturbed, non-traumatized muscle tissue.

In this study, 8 dogs were given, by the intramuscular injection, 5 mg/kg ketamine (Ketanest™, Parke-Davis, Germany) and 2 mg/kg xylazine (Rompun™, Bayer, Germany) for induction of anesthesia 30 minutes prior to endotracheal intubation. Mechanical ventilation was performed with 70% nitrous oxide in oxygen and 1.0% isoflurane. Ventilation was set to maintain end-tidal $pCO_2$ between 34 and 38 mm Hg.

The left femoral artery was cannulated for invasive measuring of arterial blood pressure and blood sampling. A 7-Swan-Ganz catheter was placed in the pulmonary artery via the right femoral vein for monitoring pulmonary artery pressure, central venous pressure and pulmonary capillary wedge pressure. A 3 mm catheter was placed in the right external jugular vein and the left femoral vein for blood exchange, and for polymerized hemoglobin solution infusion.

Following surgical preparation, anesthesia was maintained by continuous infusion of 0.025 mg/kg/hr fentanyl (Janssen, Germany) and 0.4 mg/kg/hr midazolam (Dormicum™, Roche, Germany). Muscle relaxation was achieved with 0.2 mg/kg/hr vecuronium (Norcuron™, Organon, Germany). Ventilation was set at 30% oxygen in air.

The dogs were allowed to equilibrate for 40 minutes before taking baseline readings. Following baseline readings, each dog was isovolemically hemodiluted with

hetastarch from a baseline hematocrit of about 35-45% to a hematocrit of about 25%, and then step-wise in about 5% increments, to final hematocrits of about 10%. At hematocrits of about 25%, 20%, 15% and 10%, associated hemodynamic and tissue oxygen partial pressures were measured.

After achieving hematocrits of about 10%, which was equivalent to a RBC hemoglobin concentration in the blood of about 3 g Hb/dL of blood, each dog was then infused with a polymerized hemoglobin solution (HBOC-201, also known as Hemopure 2™ Solution, Biopure Corporation, Boston, MA) in three incremental doses sufficient to raise the measured total hemoglobin (Hb from RBCs plus Hb from polymerized Hb solution) by about 0.6-1.0 g/dL per dose. Further description of Hemopure 2™ Solution is provided in the previous Examples.

All parameters were recorded after an equilibration period of 20 minutes. The time periods between the respective total hemoglobin levels was 60 minutes.

Figure 5 shows that the infusion of polymerized hemoglobin solution substantially increased regional muscle tissue oxygen tensions in anemic dogs, after the first dose of polymerized hemoglobin solution, from a mean $pO_2$ of 16 torr, associated with a RBC hemoglobin concentration of 3.0 g/dL, to a normal mean $pO_2$ of 35 torr by increasing total hemoglobin concentration by about 0.6 g/dL from the infusion of polymerized hemoglobin solution. The experimental dogs of this study had mean muscle tissue oxygen tensions of 33 torr, prior to hemodilution, which was associated with a RBC hemoglobin concentration of about 15.8 g/dL. Consequently, this study demonstrated that reduced muscle tissue oxygen tensions, which resulted from decreased availability of RBCs to transfer oxygen to the tissue,

can be improved and even restored to normal values, or above normal values, by infusing small amounts of hemoglobin into the circulatory systems of the animals. For instance, Figure 5 demonstrates that an increase in total hemoglobin of about 0.6 g Hb/dL plasma, from an infusion of polymerized hemoglobin solution, raised tissue oxygen tension by 19 torr, which was equivalent to the reduction in tissue oxygen tension associated with an decrease in RBC hemoglobin concentration of about 12.8 g Hb/dL plasma from hemodilution.

## Example 10
## Study of Tissue Oxygenation Distal to an Arterial RBC Flow Blockage

In this study, tissue oxygen tensions were measured in the hind limb muscle (m. gastrocnemius) at points distal to a 90-93% femoral artery stenosis in a Control Group (6-dogs) and a 94% femoral artery stenosis in Experimental Group A (7-dogs), following post-stenotic infusion of increasing levels of polymerized hemoglobin solution (Hemopure 2™ Solution, Biopure Corporation, Boston, MA). This study also included measurement of tissue oxygen tensions in the hind limb muscle at points distal to a 94% femoral artery stenosis in Experimental Group B (6-dogs), in which polymerized hemoglobin solution (HBOC-201) was infused prior to inducing the stenosis.

All parameters were recorded at baseline, after an equilibration period of 30 minutes following stenosis, 45 minutes after stenosis (Experimental Group B only) and 15 minutes after dosing with polymerized hemoglobin solution or hetastarch (2-hydroxyethyl ether) (Control Group and Experimental Group A only).

The dogs in the Control and Experimental Groups were anesthetized and monitored as described in Example 9. Following induction of anesthesia, baseline measurements were recorded. Baseline regional tissue oxygen tensions for the hind limb muscle of the Control Group and Experimental Group A are provided in Figure 6.

Each of the dogs of Experimental Group B were then intravenously infused with an amount of polymerized hemoglobin solution sufficient to increase the measured total hemoglobin in each dog (Hb from RBCs plus Hb from polymerized Hb solution in the plasma) by about 2.0 g/dL. The conditions of the Group B dogs were subsequently allowed to equilibrate for about 15 to about 30 minutes and tissue oxygen tensions were recorded as baseline values for Experimental Group B (Figure 7).

The femoral artery, for one hind leg of each dog in each group, was then surgically exposed and clamped with a variable arterial clamp until blood flow was reduced by approximately 90-95%. Blood flow was measured by a circumferential flow probe located distal to the stenosis. Mean regional tissue oxygen tensions, for the hind limb muscles distal to the stenosis in the dogs of the Control Group and Experimental Group A, and of Experimental Group B, 30 minutes after stenosis, are provided in Figures 2 and 3, respectively. These figures show a severe equivalent decrease in tissue oxygen tensions ($pO_2$ levels), resulting in regional hypoxia in the distal hind limb muscle, for the dogs of the Control Group and Experimental Group A (Figure 6). Specifically, as shown in Figure 2, the mean tissue oxygen tension, for the Control Group, decreased from a mean baseline value of 23 $\pm$ 2.2 torr to a mean post-stenotic value of 8 $\pm$ 0.9 torr. Further, as shown in Figure 6, the mean tissue oxygen tension, for Experimental Group A, decreased from

a mean baseline value of 27 $\pm$ 2.9 torr to a mean post-stenotic value of 11 $\pm$ 1.1 torr. In addition, at this time the distal muscle tissue for dogs of the Control Group and Experimental Group A appeared pale gray in color.

However for the dogs of Experimental Group B, which were infused with polymerized hemoglobin solution prior to inducing the 95% stenosis, at 30 minutes post-stenosis no significant decrease in mean muscle tissue oxygen tension was observed. As shown in Figure 7, the mean tissue oxygen tension for Experimental Group B decreased from a mean baseline value of 35 $\pm$ 6.9 torr to a mean post-stenotic value of 32 $\pm$ 4.5 torr.

Furthermore, 45 minutes after stenosis, the mean tissue oxygen tension, for Experimental Group B, of 36 $\pm$ 4.5 torr was not significantly different from the baseline value.

The results in Figures 6 and 7 show that induction of a "$\geq$ 90%" stenosis in an animal, created a severe hypoxic condition in tissue distal to the stenosis, except where the animal was prophylactically administered polymerized hemoglobin solution before inducing the stenosis. As shown in Figure 7, animals, which were prophylactically administered polymerized hemoglobin solution, maintained normal tissue oxygen tensions in muscle tissue distal to the stenosis, thus demonstrating the efficacy of the prophylactic administration of hemoglobin in preventing tissue hypoxia subsequent to a partial blockage of RBC flow to tissue.

Subsequently to the 30 minute post-stenotic oxygen tension measurements, each dog in the Control Group was then infused with a hetastarch in two incremental doses of 200 mL, which generally corresponds in volume to the volume of polymerized hemoglobin solution needed to raise total Hb in a dog by about 0.5 to about 0.7 g/dL per

dose. Concurrently, each dog in Experimental Group A was infused with polymerized hemoglobin solution in two incremental doses sufficient to raise the measured total hemoglobin in each dog (Hb from RBCs plus Hb from polymerized Hb solution) by about 0.5 to about 0.7 g/dL per dose.

Post-infusion regional tissue oxygen tensions for the stenotic hind limb muscles of the control group, for 200 mL and 400 mL hetastarch infusions, are provided in Figure 2. The mean tissue oxygen tensions observed were 10 ± 1.6 torr for the 200 mL hetastarch infusion and 10 ± 1.2 torr for the 400 mL hetastarch infusion. This figure shows that the post-stenotic infusion of hetastarch did not improve tissue oxygenation distal to the stenosis, as compared to the post-stenosis value of 8 ± 0.9 torr with the distal hind limb muscle remaining hypoxic and pale gray in color.

Post-infusion regional tissue oxygen tensions for the stenotic hind limb muscles of Experimental Group A, for 0.5 g/dL and 1.2 g/dL Hb solution infusions, are also provided in Figure 2. The mean tissue oxygen tensions observed were 20 ± 2.4 torr, associated with an increase in plasma Hb (and total Hb) of 0.5 g/dL, and 29 ± 2.8 torr, associated with an increase in plasma Hb (and total Hb) of 1.2 g/dL. This figure shows that the post-stenotic infusion of hemoglobin solution significantly increased mean tissue oxygen tensions for the hind limb muscle distal to the stenosis, as compared to the post-stenosis mean oxygen tension of 11 ± 1.1 torr, thus alleviating the hypoxic condition.

Improved tissue oxygenation was also demonstrated by a color change of the stenotic muscle from pale gray to a reddish color following hemoglobin solution infusion.

There were no significant differenced between baseline or post-stenotic tissue oxygen tensions when

comparing the control group and Experimental Group A. However, there were highly significant increases in tissue oxygen tension in Experimental Group A after the first Hb dose ($p<0.01$) and after the second Hb dose ($p<0.001$) when compared to the control group which received equivalent volumes of hetastarch.

A comparison of the mean oxygen tensions are provided in Figure 6, which shows the relative efficacy of treating hypoxic tissue, distal to a stenosis, with a non-oxygen bearing plasma expander, specifically hetastarch, as compared to treatment with a polymerized hemoglobin blood-substitute. Demonstrated therein, infusion of hetastarch did not improve mean tissue oxygen tension, in muscle tissue distal to a stenosis. In contrast, infusion of a polymerized hemoglobin solution significantly improved mean muscle tissue oxygen tension, in muscle tissue distal to a stenosis, to a normal value when compared to baseline.

## Example 11

### Study of Hemoglobin Solution Flow in Microvasculature Having a RBC Flow Blockage

Following induction of anesthesia, the abdomen of a Sprague-Dawley rat was surgically opened to expose the small intestines and associated mesentery. Microcirculation within the mesentery was then observed under a videomicroscope. Identified within the mesentery was a capillary with a thrombosis, with an associated complete obstruction of RBC flow. Measurement of RBC flow through this capillary gave a doppler value of zero, using an optical doppler velocimeter (Texas A&M Microvascular Research Inst.), showing no RBC movement through this capillary.

Polymerized hemoglobin solution (HBOC-201, Biopure Corporation, Boston, MA) was labeled with a fluorescent

dye, specifically fluorescein isothiocyanate and then intravenously injected into the rat at a location distant from the abdominal cavity.

The hemoglobin in the polymerized hemoglobin solution was labeled with fluorescein isothicyanate (hereinafter "FITC") by employing a modification of the method described by Wilderspin in *Anal. biochem.*, 132: 449 (1982) and Ohshiata in *Anal. biochem.*, 215: 17-23 (1993). A stock solution of FITC label was prepared by dissolving 6.6 g of FITC in 615 mL of 100 mM borate buffer (pH 9.5). Polymerized hemoglobin solution (923 mL at 13 g Hb/dL) was loaded into a nitrogen flushed vessel equilibrated with 512 mL of borate buffer. The FITC/borate buffer was then added at 11.8 mL/min through a static mixer loop to the hemoglobin/borate mixture. The reaction proceeded for 2 hours at room temperature in a nitrogen environment with continuous stirring. Residual FITC was removed by diafiltration with a 30 kD, membrane (Millipore Pellicon, 5 sq. ft.) for seven volume exchanges with a lactate storage solution (pH 7.7). After the last exchange, the system was concentrated to 8.6 g/dL hemoglobin and the material was aliquoted into nitrogen evacuated 10 mL Vacutainer tubes with 60 mL syringes using anaerobic techniques. The tubes were wrapped in tin foil and stored at 4°C until use. A 10:1 molar ratio of FITC:Hb, used in the reaction, gave a 5:1 ratio of FITC:Hb in the labeled Hb product.

Following injection of the labeled hemoglobin, within about one minute, labelled hemoglobin was then observed entering and flowing through the thrombotic capillary, past the stagnant and stacked aggregation of red blood cells.

The results of this study demonstrate that hemoglobin can flow through microvasculature, through which RBC flow is restricted or precluded, thereby

allowing increased oxygen transport by the hemoglobin to tissue associated with the thrombotic capillary wherein there is no RBC flow.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other such equivalents are intended to be encompassed by the following claims.

## CLAIMS

The invention claimed is:

1. A method for producing a purified hemoglobin product substantially free of other blood protein components and contaminants, comprising subjecting a red blood cell fraction containing hemoglobin to a chromatographic column and eluting said purified hemoglobin product with a pH gradient, thereby forming a hemoglobin eluate.

2. The method of Claim 1, wherein the red blood cell fraction containing hemoglobin is obtained by a method comprising the steps of:
   a) mixing blood with an anticoagulant to form a blood solution;
   b) washing the red blood cells in the blood solution;
   c) separating the washed red blood cells from white blood cells; and
   d) disrupting the separated red blood cells, thereby obtaining a mixture of proteins and cell debris that comprises a red blood cell fraction containing hemoglobin.

3. The method of Claim 2, further comprising the steps of:
   a) separating the proteins from the cell debris, thereby obtaining a protein extract;
   b) fractionating the protein extract to remove high molecular weight proteins, thereby obtaining a low molecular weight protein fraction containing hemoglobin; and

c) concentrating the hemoglobin in the low molecular weight protein fraction by ultrafiltration, thereby forming a purified red blood cell fraction containing hemoglobin.

4. The method of Claim 3, further comprising the steps of:

a) deoxygenating the hemoglobin in the hemoglobin eluate to form a deoxygenated hemoglobin solution;

b) mixing the deoxygenated hemoglobin solution with a first reducing agent, to form an oxidation-stabilized, deoxygenated hemoglobin solution;

c) mixing said oxidation-stabilized, deoxygenated hemoglobin solution with a cross-linking agent to form a polymerization reaction mixture;

d) polymerizing the polymerization reaction mixture, thereby forming a stable polymerized hemoglobin solution; and

e) purifying the polymerized hemoglobin solution from non-polymerized hemoglobin in the presence of a physiologic solution and a second reducing agent, whereby the polymerized hemoglobin solution is made physiologically acceptable, and whereby the reducing agent scavenges oxygen, thereby forming a stable, polymerized, purified hemoglobin product.

5. A method of Claim 4, wherein the polymerized hemoglobin solution is purified from non-polymerized hemoglobin by diafiltration using an ultrafiltration membrane.

6. A method of Claim 4, wherein the polymerization reaction mixture is stabilized in its molecular weight distribution by chemically reducing the polymerization reaction mixture.

7. A method of Claim 1, wherein the red blood cell fraction is subjected to a chromatographic column that comprises a packing for ion-exchange affinity separation of endotoxin from hemoglobin.

8. A method of Claim 1, further comprising the steps of:
   a) mixing blood containing red blood cells with an anticoagulant to form a blood solution;
   b) diluting the blood solution with an isotonic solution;
   c) filtering said diluted blood solution to separate smaller plasma components from the red blood cells;
   d) centrifuging said filtered blood solution to separate white blood cells from the red blood cells;
   e) directing the red blood cells against a surface to tear the cell membranes of at least a portion of the red blood cells to form a hemoglobin solution;
   f) ultrafiltering the hemoglobin solution to remove large cell debris from the hemoglobin solution; and
   g) ultrafiltering the hemoglobin solution to remove small non-hemoglobin components from the hemoglobin solution, thereby producing a purified red blood cell fraction containing hemoglobin.

9. A method of Claim 8, further comprising the steps of:
   a) deoxygenating the hemoglobin eluate to form a deoxygenated hemoglobin solution;
   b) mixing said deoxygenated hemoglobin solution with a reducing agent, to form an oxidation-stabilized, deoxygenated hemoglobin solution;
   c) mixing said oxidation-stabilized, deoxygenated hemoglobin solution with a cross-linking agent to form a polymerization reaction mixture;
   d) polymerizing the polymerization reaction mixture, thereby forming a polymerized hemoglobin solution;
   e) contacting the polymerized hemoglobin solution with an alkaline solution, whereby the polymerized hemoglobin solution is basified;
   f) contacting the basified polymerized hemoglobin solution with sodium borohydride, whereby unstable bonds are reduced, thereby forming a stable polymerized hemoglobin solution;
   g) diafiltering the stable polymerized hemoglobin solution with a physiologic solution, whereby the polymerized hemoglobin solution is made physiologically acceptable, thereby forming ultrapure, stable, polymerized hemoglobin product; and
   h) filtering said ultrapure stable polymerized hemoglobin product to separate tetrameric hemoglobin from said ultrapure stable polymerized hemoglobin product.

10. A method for preserving the oxidation stability of a hemoglobin product comprising maintaining the hemoglobin product in an atmosphere substantially free of oxygen.

11. A method for producing a polymerized hemoglobin solution from hemoglobin contained in a hemoglobin solution, comprising the steps of:
    a) mixing said hemoglobin solution with a reducing agent, whereby the hemoglobin solution is deoxygenated, thereby forming an oxygen-stabilized, deoxygenated hemoglobin solution;
    b) mixing said mixing said oxygen-stabilized, deoxygenated hemoglobin solution with a cross-linking agent, thereby forming a polymerization reaction mixture; and
    c) polymerizing the polymerization reaction mixture, thereby forming a polymerized hemoglobin solution.

12. A method for producing a polymerized hemoglobin solution from hemoglobin contained in a hemoglobin solution, comprising the steps of:
    a) deoxygenating said hemoglobin solution, thereby forming deoxygenated hemoglobin solution;
    b) mixing said deoxygenated hemoglobin solution with a first reducing agent, thereby forming an oxygen-stabilized, deoxygenated hemoglobin solution;
    c) mixing said mixing said oxygen-stabilized, deoxygenated hemoglobin solution with a cross-linking agent, thereby forming a polymerization reaction mixture; and
    d) polymerizing the polymerization reaction mixture, thereby forming a polymerized hemoglobin solution.

13. A method of Claim 12, wherein the cross-linking agent is a dialdehyde, and the polymerization reaction mixture is polymerized with heat.

14. A method of Claim 13, further comprising the steps of:
    a) contacting the polymerized hemoglobin solution with an alkaline solution, whereby the polymerized hemoglobin solution is basified;
    b) contacting the basified polymerized hemoglobin solution with a second reducing agent, whereby a stable, reduced polymerized hemoglobin solution is formed; and
    c) diafiltering the stable, reduced polymerized hemoglobin solution with a first physiologic solution, having a pH of 7.9 or lower, whereby the reduced polymerized hemoglobin solution is made physiologically acceptable, thereby forming a stable, polymerized hemoglobin solution.

15. A method of Claim 14, further comprising the step of diafiltering the stable polymerized hemoglobin solution with a second physiologic solution, having a pH between 7.6 and 7.9, against a filter suitable to separate non-polymerized hemoglobin from polymerized hemoglobin.

16. The method of Claim 15, wherein the mammalian hemoglobin is bovine hemoglobin; wherein the first reducing agent is N-acetyl-L-cysteine; wherein the dialdehyde of is glutaraldehyde; wherein the alkaline solution is an alkaline borate buffer; wherein the second reducing agent is a sodium borohydride; and wherein the first physiologic solution contains 27 mM sodium lactate, 12 mM NAC, 115 mM NaCl, 4 mM KCl, and 1.36 mM $CaCl_2$ and has a pH of about 5.

17. A stable polymerized hemoglobin solution comprising:
    a) polymerized hemoglobin; and
    b) a reducing agent, wherein said reducing agent stabilizes said polymerized hemoglobin.

18. The stable polymerized hemoglobin solution of Claim 17, wherein the reducing agent is selected from the group consisting of: N-acetyl-L-cysteine, D,L-cysteine, glutathione, γ-glutamyl-cysteine, 2-mercaptoethanol, 2,3-dimercapto-1-proponal, dithioerythritol, dithiothreitol, thioglycolate, 1,4-butanedithiol, citrate salt; citric acid, reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, and reduced flavin adenine dinucleotide.

19. A stable polymerized hemoglobin solution produced by the method of Claim 1.

20. A stable polymerized hemoglobin solution, comprising mammalian hemoglobin, with a concentration between 120 to 140 grams of hemoglobin per liter of solution, having the following characteristics:
    a) a methemoglobin content of less than 15 percent by weight;
    b) an oxyhemoglobin content of less than or equal to 10 percent by weight;
    c) an endotoxin concentration of less than 0.5 Endotoxin units per milliliter;
    d) less than, or equal to, 15 percent by weight of the polymerized hemoglobin having a molecular weight over 500,000 Daltons; and
    e) less than, or equal to, 5 percent by weight of the hemoglobin being unstabilized tetrameric hemoglobin.

21. A stable polymerized hemoglobin solution, comprising mammalian hemoglobin, with a concentration between 10 to 250 grams of hemoglobin per liter of solution, having the following characteristics:
    a) a methemoglobin content of less than 15 percent by weight;
    b) an oxyhemoglobin content of less than or equal to 10 percent by weight;
    c) an endotoxin concentration of less than 0.5 Endotoxin units per milliliter;
    d) less than, or equal to, 15 percent by weight of the polymerized hemoglobin having a molecular weight over 500,000 Daltons;
    e) less than, or equal to, 10 percent by weight of the polymerized hemoglobin having a molecular weight under 65,000 Daltons; and
    f) less than, or equal to, 5 percent by weight of the hemoglobin having a molecular weight under 32,000 Daltons.

22. Use of the stable polymerized hemoglobin solution of Claim 19, 20, or 21, for the manufacture of a medicament for increasing tissue oxygenation in the tissue of a vertebrate having reduced red blood cell flow.

23. Use of the stable polymerized hemoglobin solution of Claim 19, 20, or 21, for the manufacture of a medicament for increasing tissue oxygenation or preventing oxygen depletion as a prophylaxis against reduced red blood cell flow.

24. A method for increasing tissue oxygenation in the tissue of a vertebrate, wherein the tissue has reduced blood cell flow, comprising introducing into the circulatory system of the vertebrate, at least one dose of hemoglobin.

25. A method for increasing tissue oxygenation, or preventing oxygen depletion, in a vertebrate as a prophylaxis against reduced oxygenation as a consequence of a reduction in red blood cell flow in the vertebrate, comprising introducing into the circulatory system of the vertebrate at least one dose of hemoglobin.

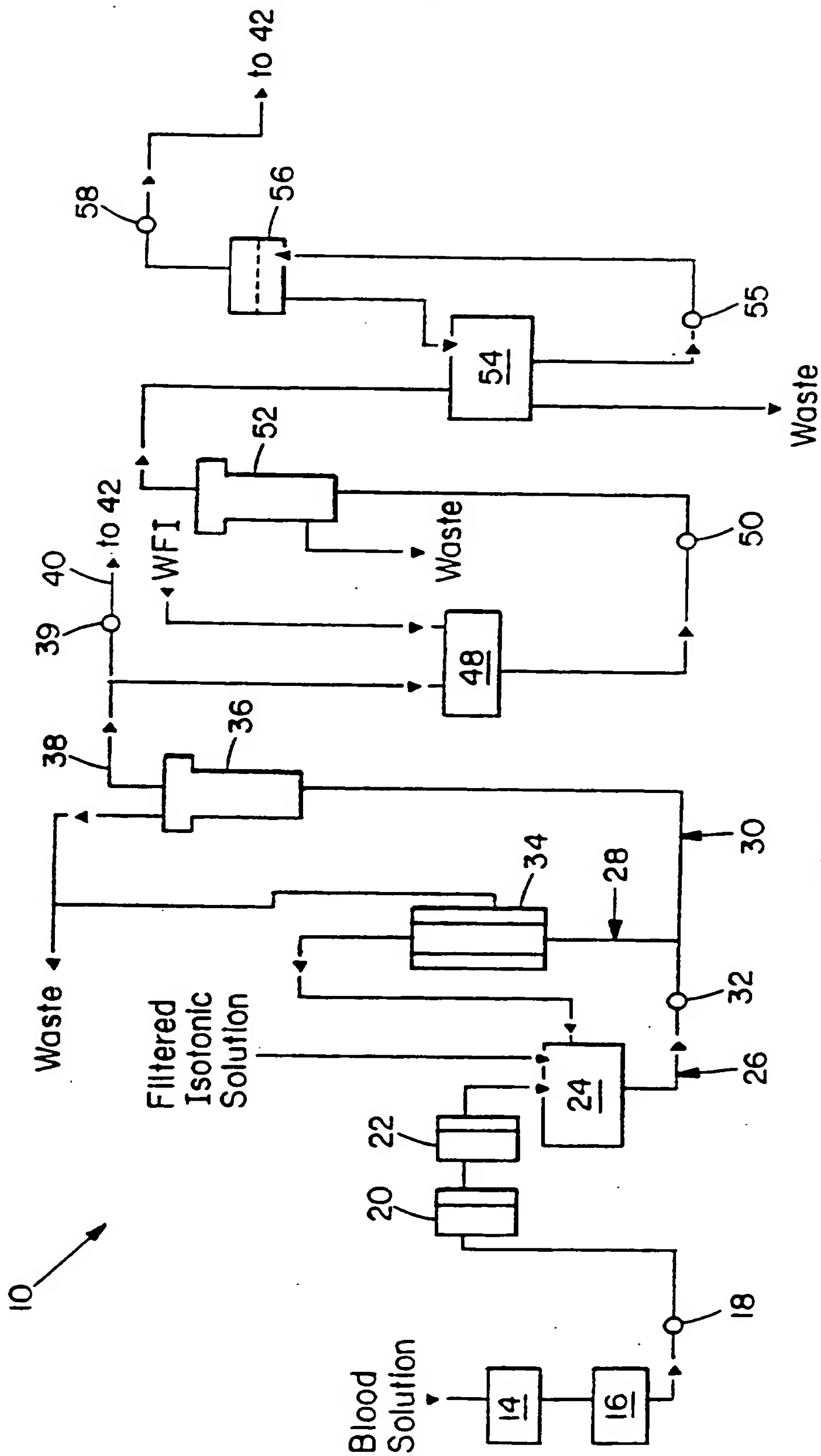

Blood Solution
14
16
18
10
20
22
24
26
Filtered Isotonic Solution
32
28
34
30
Waste
36
38
39
40
to 42
WFI
48
50
52
Waste
54
Waste
55
56
58
to 42

FIG. 1A

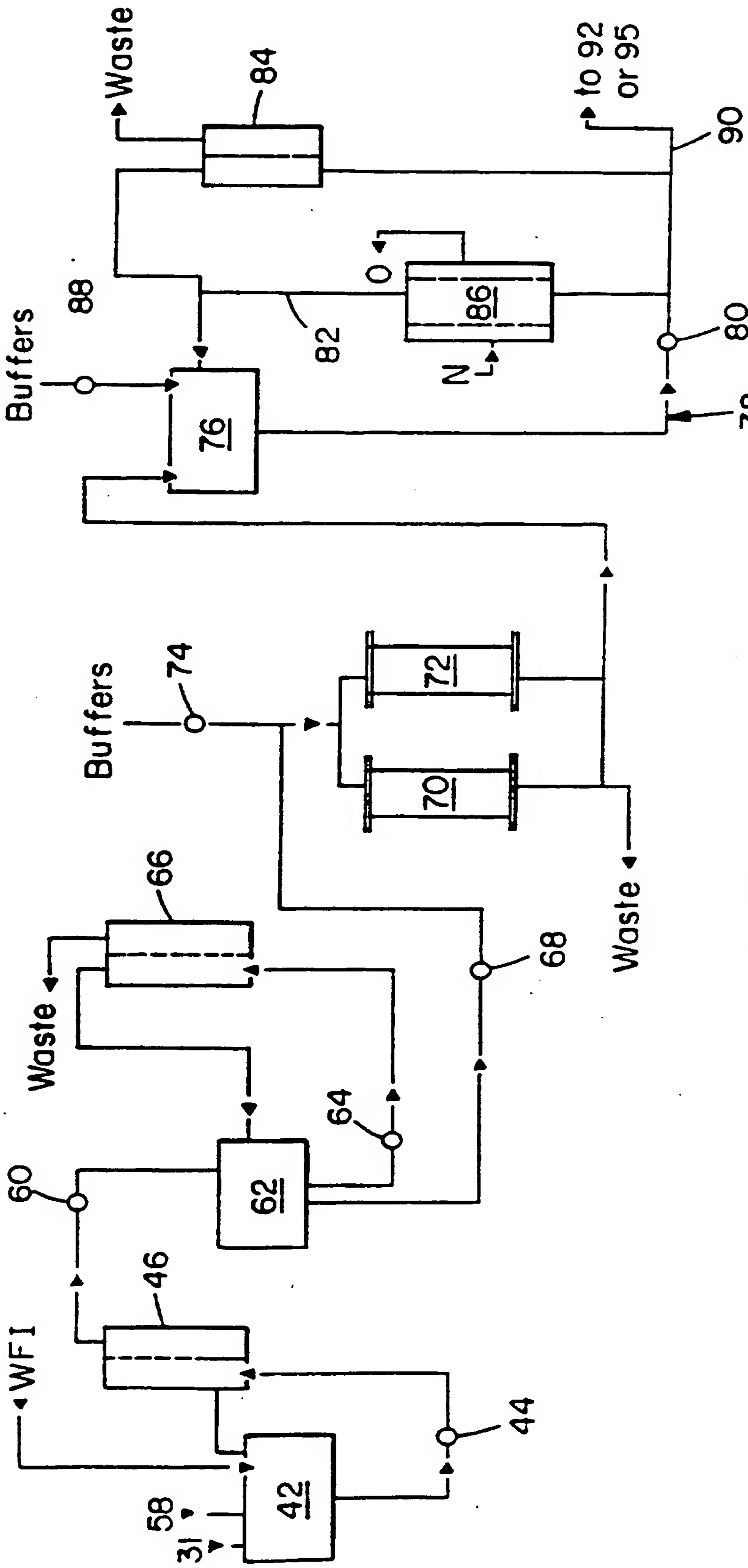
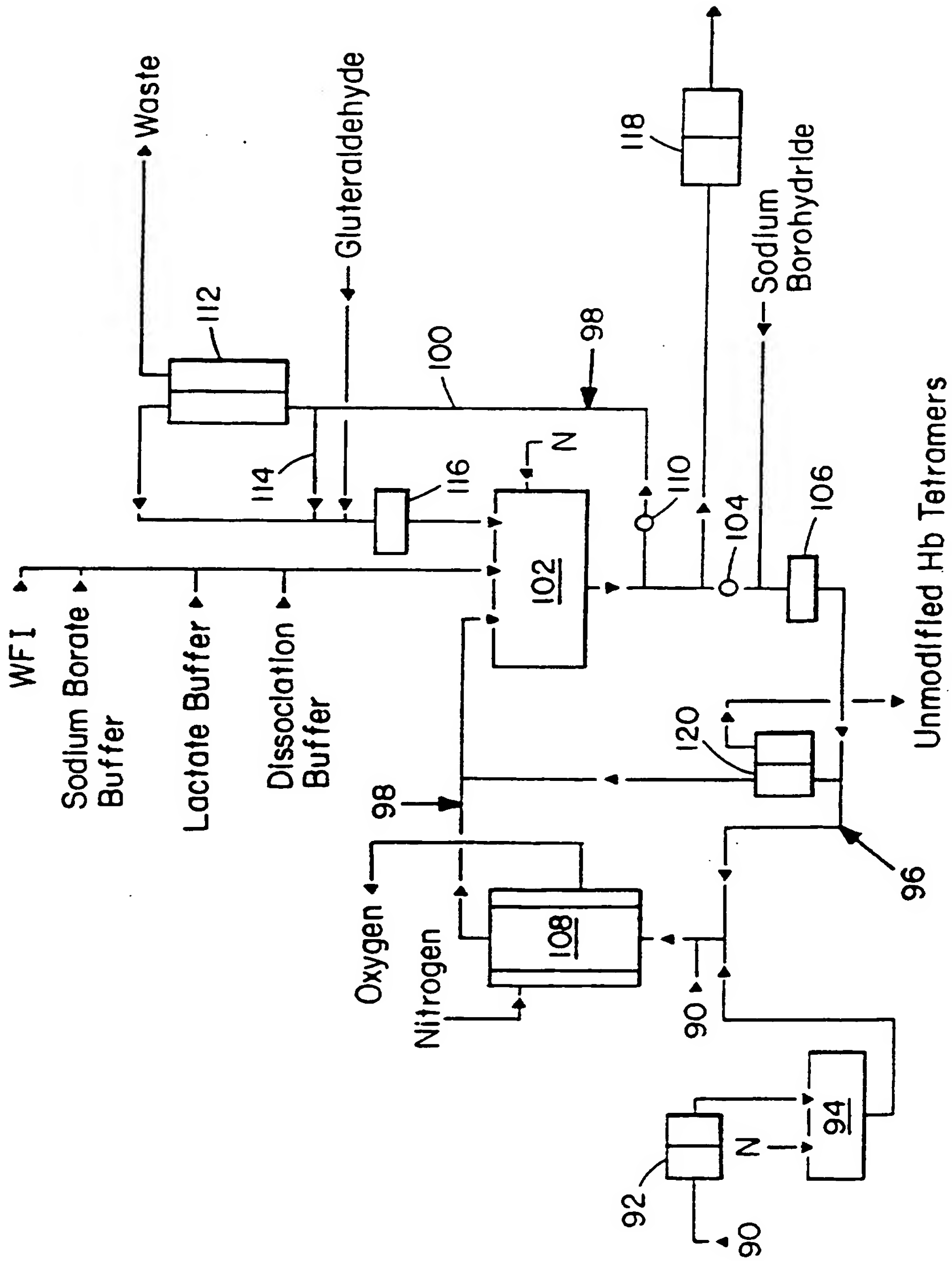

Waste
84
to 92
or 95
90
O
86
N
L
80
82
88
Buffers
76
78
Buffers
74
72
70
Waste
66
Waste
68
64
60
62
46
WFI
44
42
58
31

FIG. 1B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Gluteraldehyde
Sodium Borohydride
WFI
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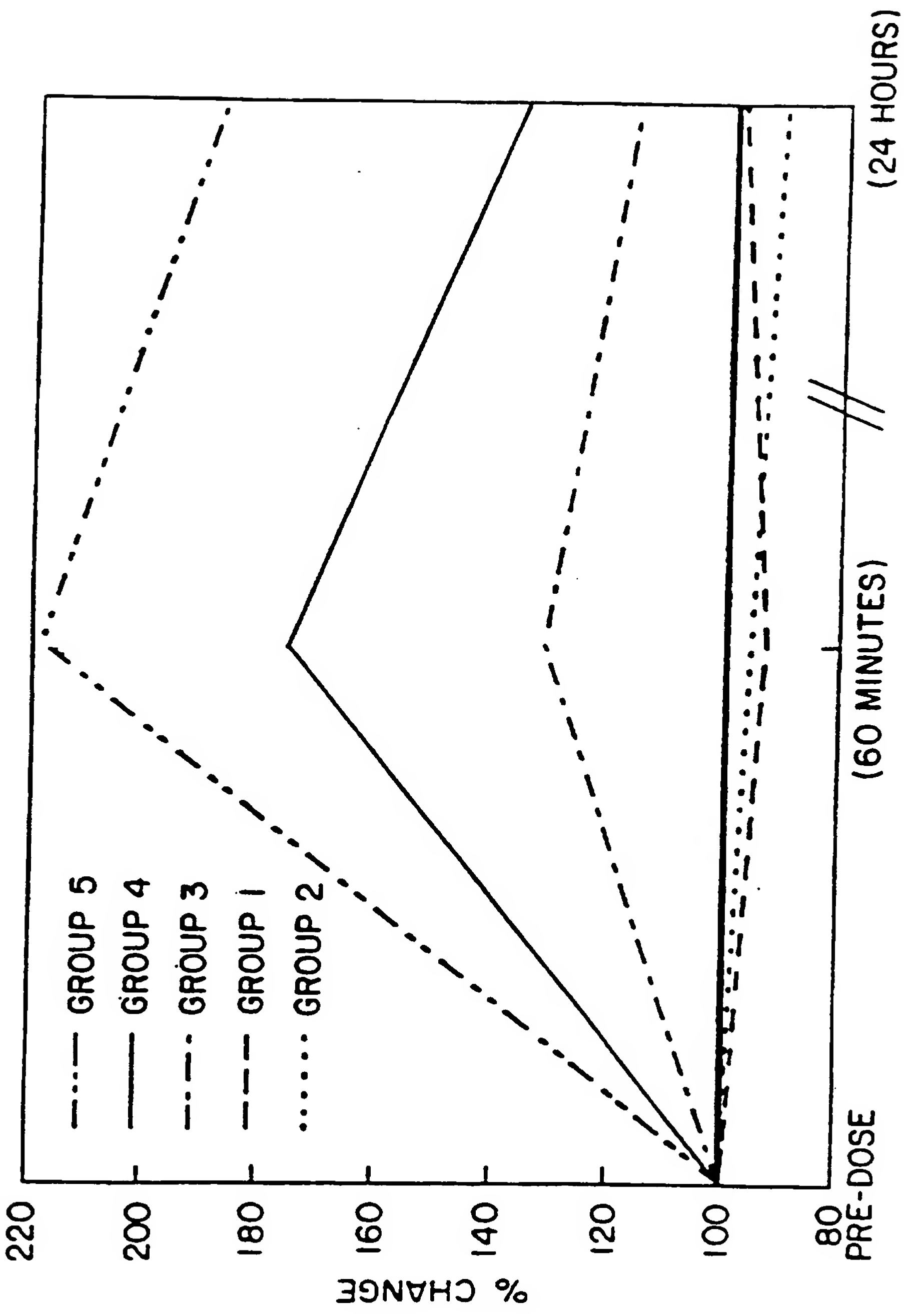
Lactate Buffer
Dissociation Buffer
Oxygen
Nitrogen
Unmodified Hb Tetramers
N
N
90
90
92
94
96
98
98
100
102
104
106
108
110
112
114
116
118
120

FIG. 1C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GROUP 4
GROUP 3
GROUP 1
GROUP 2
% CHANGE
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200
180
160
140
120
100
80
PRE-DOSE
(60 MINUTES)
(24 HOURS)

FIG. 2

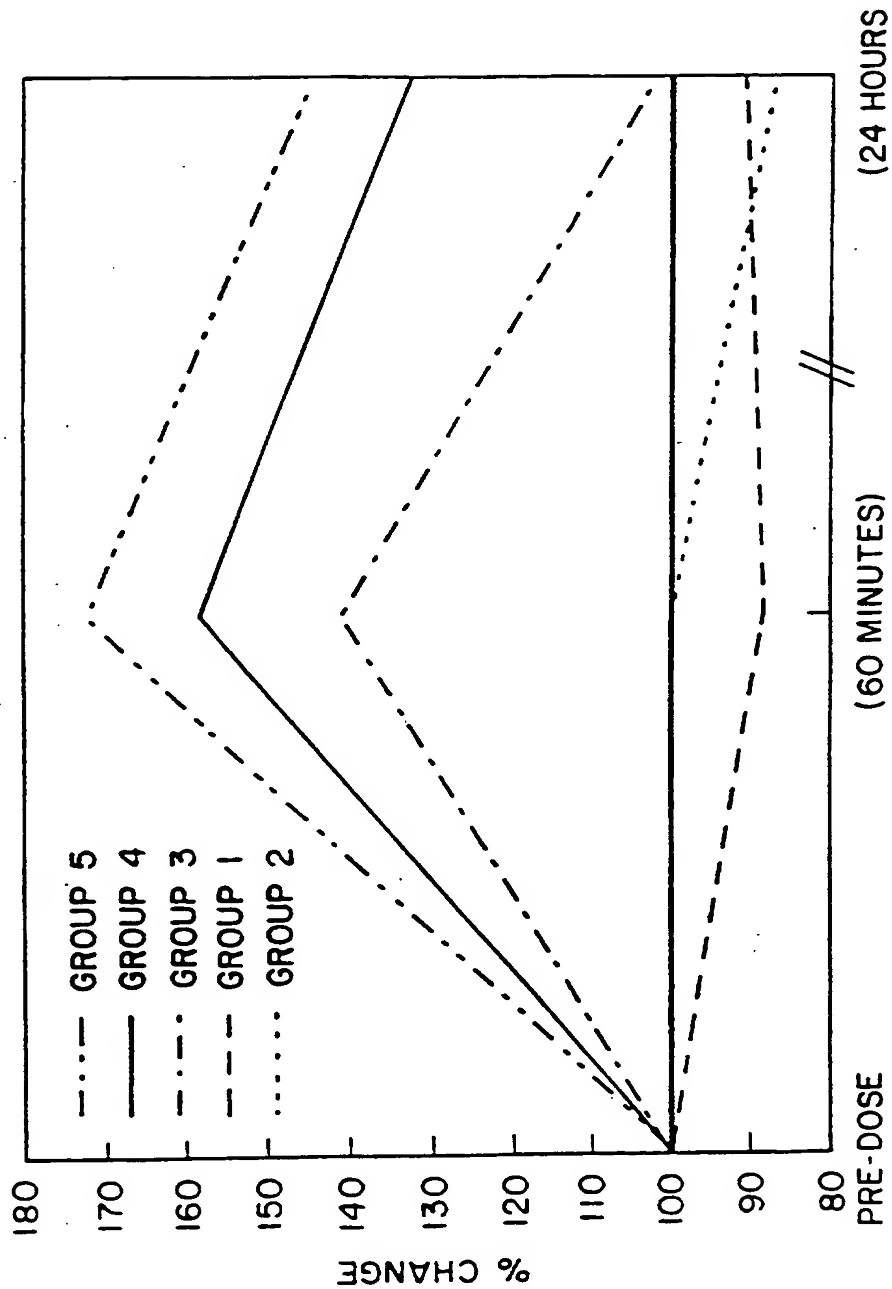
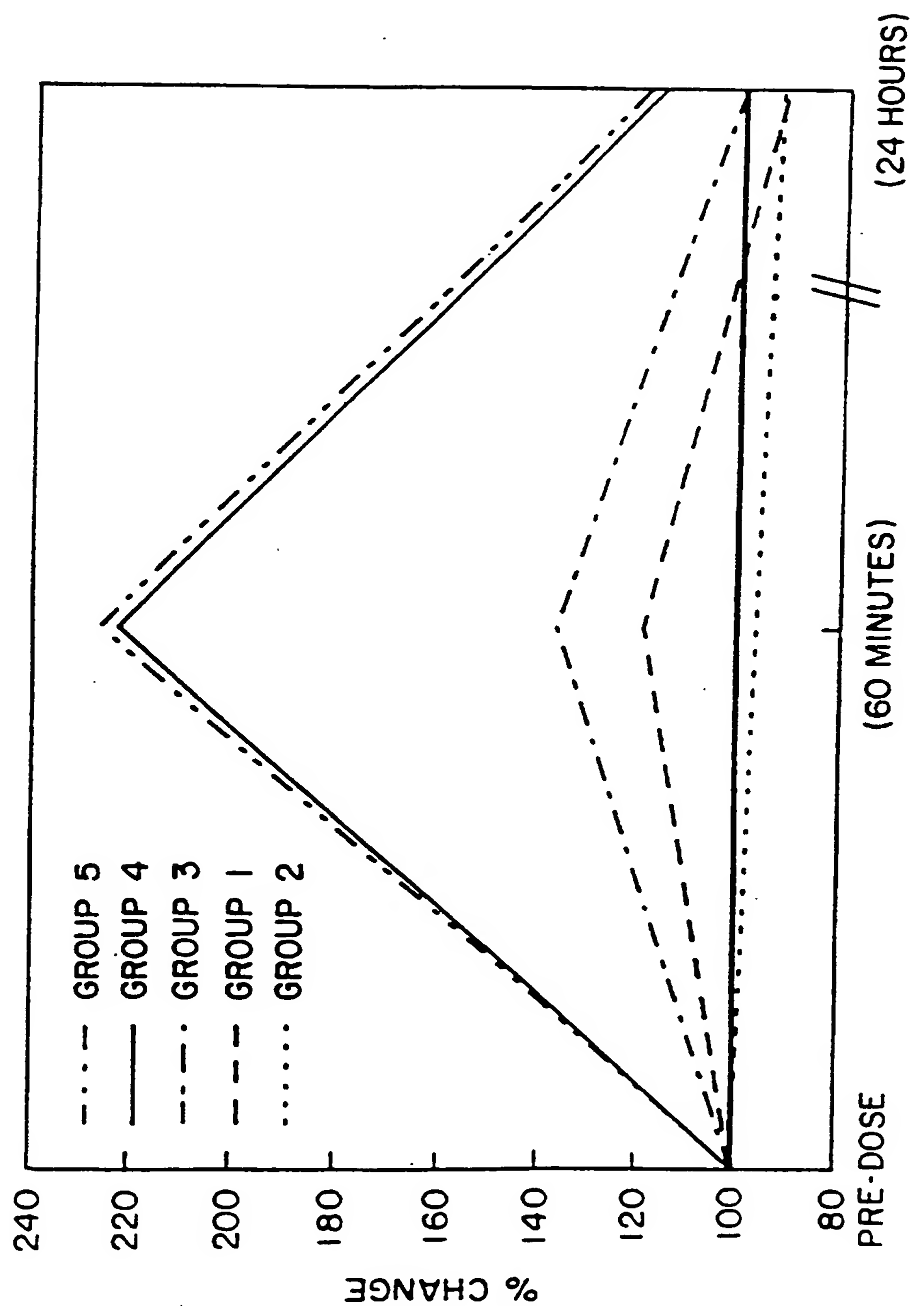

% CHANGE
180
170
160
150
140
130
120
110
100
90
80
PRE-DOSE
(60 MINUTES)
(24 HOURS)
GROUP 5
GROUP 4
GROUP 3
GROUP 1
GROUP 2

FIG. 3


GROUP 5
GROUP 4
GROUP 3
GROUP 1
GROUP 2
% CHANGE
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220
200
180
160
140
120
100
80
PRE-DOSE
(60 MINUTES)
(24 HOURS)

FIG. 4

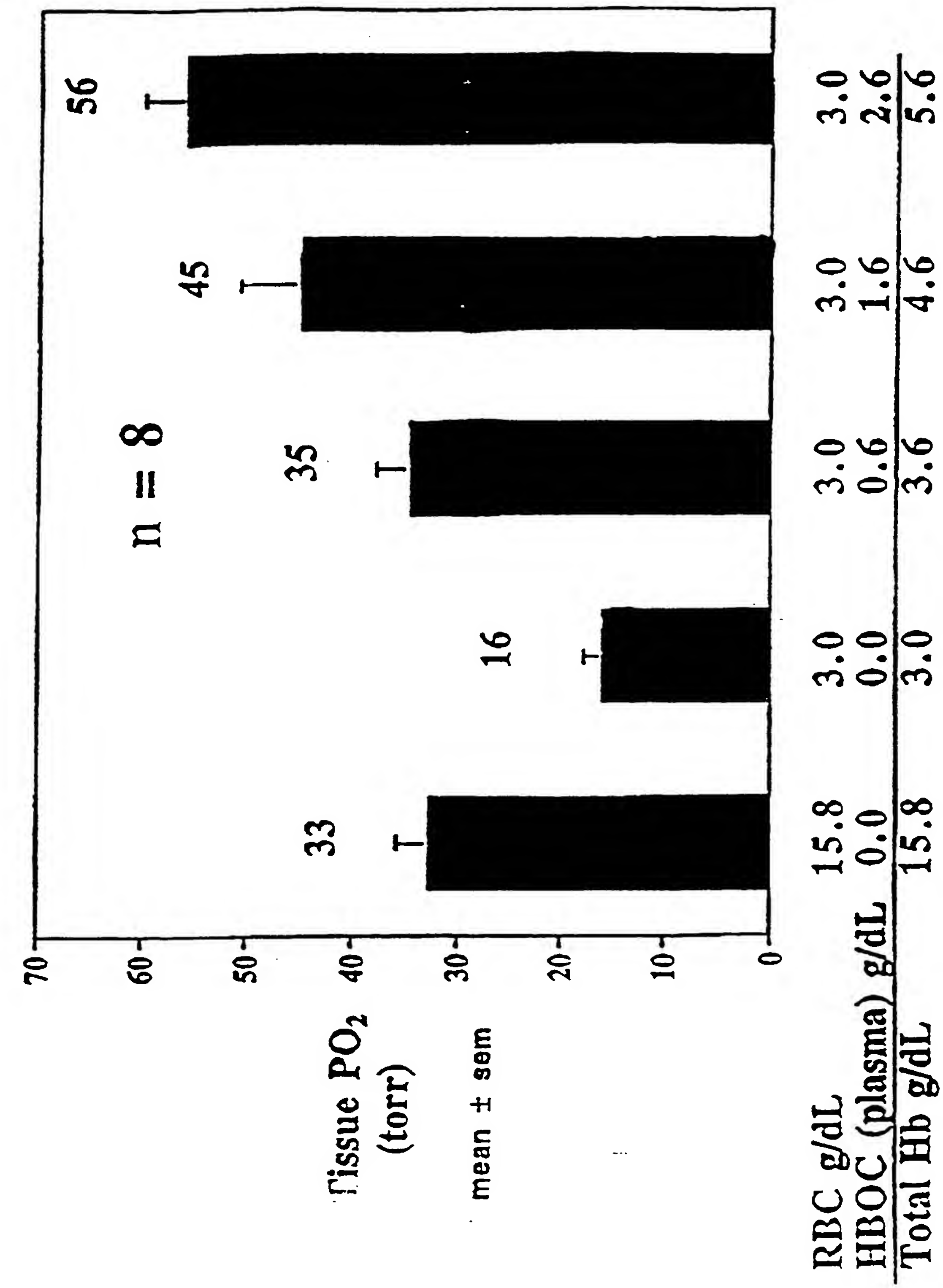
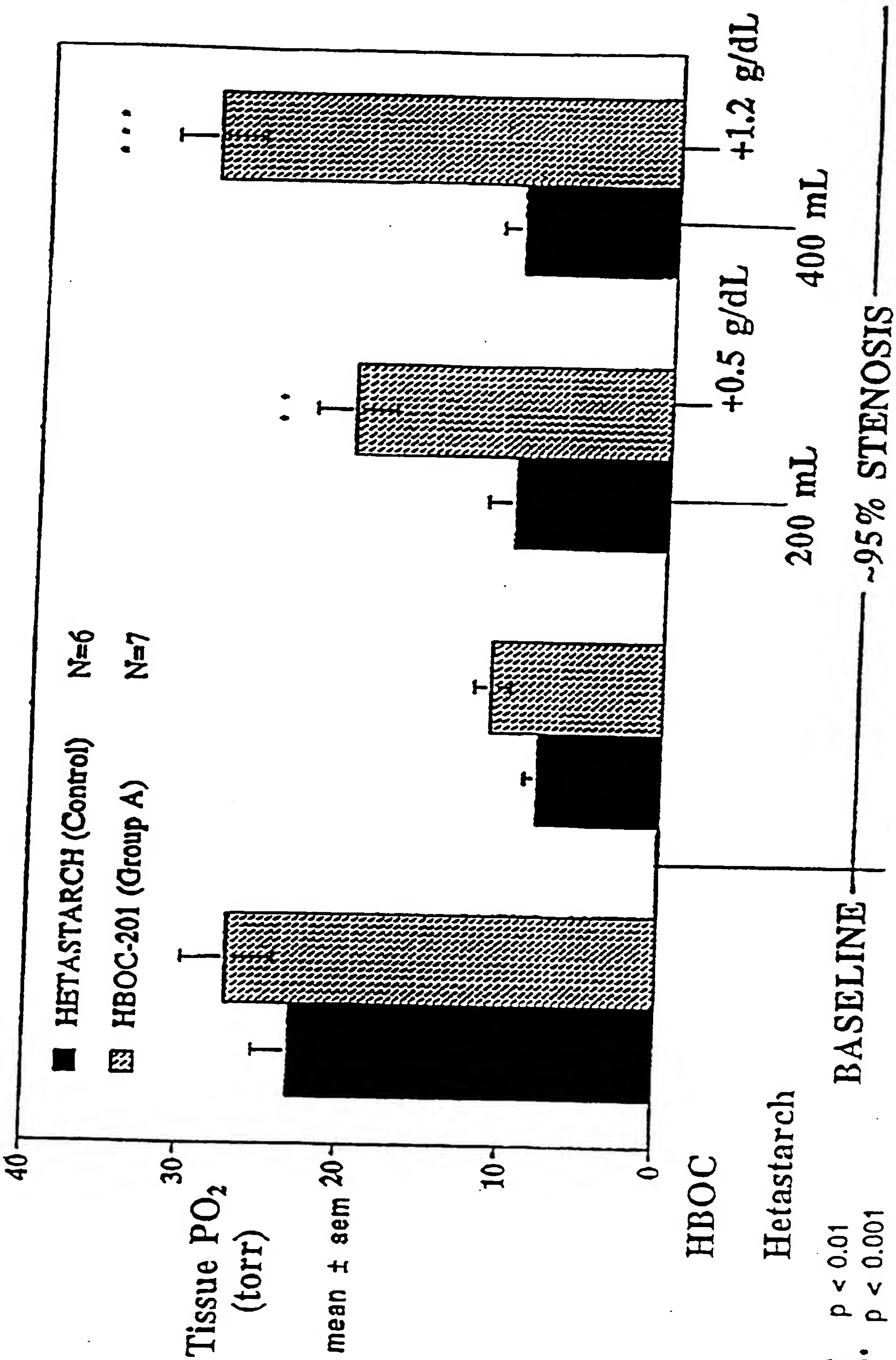
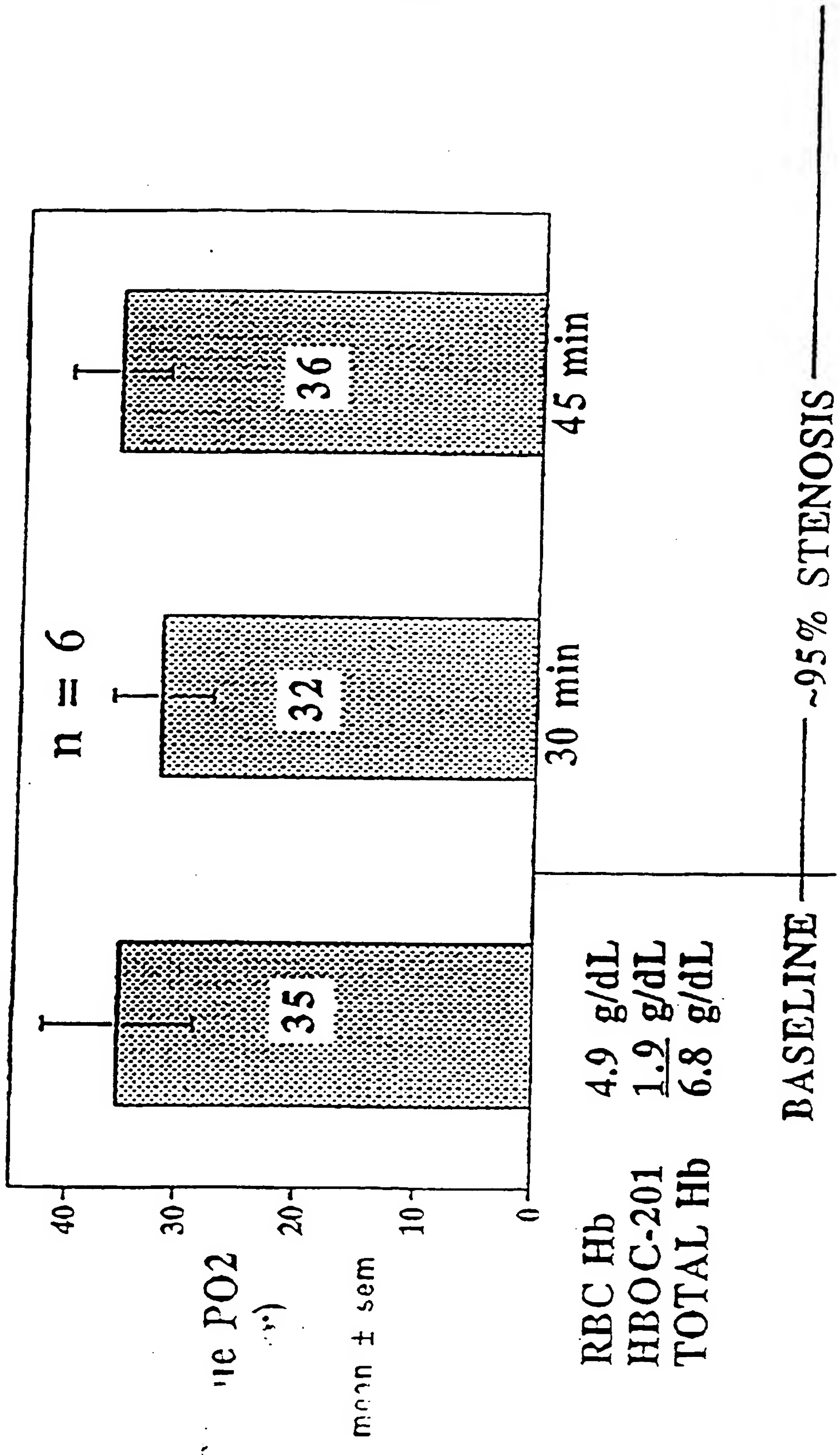

Tissue $PO_2$ (torr)
mean ± sem
n = 8
70
60
50
40
30
20
10
0
33
16
35
45
56
RBC g/dL 15.8 3.0 3.0 3.0 3.0
HBOC (plasma) g/dL 0.0 0.0 0.6 1.6 2.6
Total Hb g/dL 15.8 3.0 3.6 4.6 5.6

FIGURE 5



Tissue $PO_2$ (torr)
mean ± sem
0
10
20
30
40
HETASTARCH (Control) N=6
HBOC-201 (Group A) N=7
HBOC
Hetastarch
BASELINE
200 mL
+0.5 g/dL
400 mL
+1.2 g/dL
~95% STENOSIS
** $p < 0.01$
*** $p < 0.001$


FIGURE 6



n = 6
35
32
36
30 min
45 min
40
30
20
10
0
RBC Hb 4.9 g/dL
HBOC-201 1.9 g/dL
TOTAL Hb 6.8 g/dL
BASELINE
~95% STENOSIS


FIGURE 7

# INTERNATIONAL SEARCH REPORT

Intern nal Application No
PC1/US 96/04030

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C07K14/805 C07K1/18 A61K38/42

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO,A,88 03408 (BIOPURE CORPORATION) 19 May 1988<br>cited in the application | 1-3,5,7, 8,19-25 |
| Y | see page 23, line 26 - page 49, paragraph 3; claims | 4,6,9, 11-16 |
| X | WO,A,89 12456 (BAXTER INTERNATIONAL INC.) 28 December 1989 | 10,17,18 |
| Y | see page 3, line 26 - page 5, line 31 | 4,6,9, 11-16 |
| X | US,A,5 264 555 (R.G.L. SHORR ET AL.) 23 November 1993<br>see claims 37-64 | 1-3,7, 19-25 |

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 18 June 1996 | 27.06.96 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Ryckebosch, A |

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/ US 96/ 04030

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 24,25
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: ALTHOUGH CLAIMS 24,25 ARE DIRECTED TO A METHOD OF TREATMENT OF THE HUMAN/ANIMAL BODY THE SEARCH HAS BEEN CARRIED OUT AND BASED ON THE ALLEGED EFFECTS OF THE COMPOUND/COMPOSITION.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search :s were accompanied by the applicant's protest.

[ ] No protest accompaniec :he payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PC.,US 96/04030

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-8803408 | 19-05-88 | AU-B- | 622610 | 16-04-92 |
| | | AU-B- | 1087188 | 01-06-88 |
| | | CA-A- | 1312009 | 29-12-92 |
| | | DE-A- | 3778006 | 07-05-92 |
| | | EP-A,B | 0277289 | 10-08-88 |
| | | JP-T- | 2501067 | 12-04-90 |
| | | US-A- | 5084558 | 28-01-92 |
| | | US-A- | 5296465 | 22-03-94 |
| WO-A-8912456 | 28-12-89 | EP-A- | 0379534 | 01-08-90 |
| | | JP-T- | 3502580 | 13-06-91 |
| US-A-5264555 | 23-11-93 | AU-B- | 4638393 | 31-01-94 |
| | | EP-A- | 0654039 | 24-05-95 |
| | | WO-A- | 9401452 | 20-01-94 |